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DECEMBER 21, 2023

THE CONTROLLER OF PATENT
THE PATENT OFFICE
DELHI, MUMBAI, KOLKATA, CHENNAI

RE: OPPOSITION U/S 25(2) OF THE PATENT ACT – BY SANKALP REHABILITATION TRUST AGAINST INDIAN PATENT NO. 427560 (FORMERLY PATENT APPLICATION NUMBER 5348/DELNP/2014) DATED 30/06/2014
APPLICANT: 1. EMORY UNIVERSITY 2. GAVEGNANO, CHRISTINA 3. SCHINAZI, RAYMOND, F.

Respected Sir,

We are filing this representation by way of Post-Grant Opposition along with annexures u/s 25 (2) of the Patents Act, 1970 and Rule 55 of the Patent Rules, 2003 in Form 7.

The Learned Controller is requested to take said opposition along with annexures on record and proceed further in the matter and keep the Opponent advised of each and every step taken in the matter.

We crave the leave of the Learned Controller to submit additional documents and/or evidence to support any of the averments in the representation as may be necessitated during the future proceeding.

Lastly, we request the Learned Controller to grant an opportunity of being heard before the present Opposition is finally decided.

Thanking you,

Yours faithfully,

PRAGYA SINGH THAKUR (IN /PA – 3329)
OF RAJESHWARI AND ASSOCIATES
AGENT FOR THE OPPONENT

Encl.: As stated

C.C.: KHURANA AND KHURANA
Email: docket@khuranaandkhurana.com; info@khuranaandkhuran;

BEFORE THE CONTROLLER OF PATENTS, NEW DELHI

IN THE MATTER OF:

The Patents Act, 1970 as amended by the Patents (Amendment) Act 2005, and The Patents Rules, 2003, as amended by The Patents (Amendment) Rules, 2006

AND

IN THE MATTER of Post-Grant Opposition under Section 25(2)

AND

IN THE MATTER of Indian Patent No. 427560

(Formerly Indian Patent Application No. 5348/DELNP/2014)

IN THE MATTER OF:

SANKALP REHABILITATION TRUST **.....**OPPONENT

VS.

1. EMORY UNIVERSITY .....APPLICANT/

2. GAVEGNANO, CHRISTINA PATENTEE

3. SCHINAZI, RAYMOND, F.

POST-GRANT OPPOSITION BY SANKALP REHABILITATION TRUST

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	Complexity"; Curr HIV/AIDS Rep (2010) 7:37–43 published 27 January 2010.	
7.	**Annexure – 5:** Copy of Article Gavegnano et al., "Antiretroviral therapy in macrophages: implication for HIV eradication"; Antiviral Chemistry & Chemotherapy 2009 20: 63–78.	115-130
8.	**Annexure – 6:** Copy of duly notarized expert affidavit of Mrs. Mansi Verma	131-147
9.	**Annexure – 7:** Copy of CV of Mrs. Mansi Verma	148-152
10.	Copy of duly stamped Power of Attorney	Will follow

Dated this 21st day of December, 2023

PRAGYA SINGH THAKUR (IN /PA – 3329)
OF RAJESHWARI AND ASSOCIATES
AGENT FOR THE OPPONENT

TO

THE CONTROLLER OF PATENTS

THE PATENT OFFICE, NEW DELHI

FORM 7

THE PATENTS ACT, 1970

(39 OF 1970)

AND

THE PATENTS RULES, 2003

NOTICE OF OPPOSITION

[See Section 25(2) and rule 55A]

We, **SANKALP REHABILITATION TRUST**, having its registered office at SS Bengali Municipal School, First Floor, Thakurdwar Road, Charni Road East, Mumbai – 400002, hereby give Notice of opposition to the grant of patent in respect of Indian Patent No. 427560 (formerly patent application number 5348/DELNP/2014) dated 30/06/2014 made by **1. EMORY UNIVERSITY 2. GAVEGNANO, CHRISTINA 3. SCHINAZI, RAYMOND, F.** on the grounds:

(a) Section 25(2)(e): Lack of inventive step

(b) Section 25(2)(f): Invention is not patentable under section 3(d) and 3(e)

(c) Section 25(2)(g): The complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.

(d) Section 25(2)(h): Failure to disclose the information required by section 8 of the Patents Act.

(Detailed grounds are set out in the Opposition)

Our address for service in India is:

RAJESHWARI & ASSOCIATES, S – 357, FIRST FLOOR, NEAR HDFC BANK, PANCHSHEEL PARK, NEW DELHI – 110017, INDIA, Tel: + 91-11-41038911, Mobile No. 8368982401, Email: pragya@ralegal.co.in;

Dated this 21st day of December, 2023

PRAGYA SINGH THAKUR (IN /PA – 3329)
OF RAJESHWARI AND ASSOCIATES
AGENT FOR THE OPPONENT

TO,
THE CONTROLLER OF PATENTS
THE PATENT OFFICE, NEW DELHI

BEFORE THE CONTROLER OF PATENTS, THE PATENT OFFICE, NEW DELHI

In the matter of Section 25(2) of The Patents Act, 1970 as amended by The Patents (Amendment) Act 2005;

And

In the matter of Rule 55 of The Patents Rules 2003 as amended by the Patent (Amendment) Rules, 2006

And

In the matter of Indian Patent number 427560 (formerly patent application number 5348/DELNP/2014) dated 30/06/2014, in the name of **1. EMORY UNIVERSITY 2. GAVEGNANO, CHRISTINA 3. SCHINAZI, RAYMOND, F.**

REPRESENTATION BY:

SANKALP REHABILITATION TRUST **.....**OPPONENT

VS.

1. EMORY UNIVERSITY .....APPLICANT/
2. GAVEGNANO, CHRISTINA PATENTEE
3. SCHINAZI, RAYMOND, F.

REPRESENTATION BY WAY OF POST-GRANT OPPOSITION UNDER SECTION 25(2) OF THE PATENTS ACT, 1970

We, S**ANKALP REHABILITATION TRUST**, having its registered office at SS Bengali Municipal School, First Floor, Thakurdwar Road, Charni Road East, Mumbai – 400002, hereby submit our representation by way of opposition to the patent 427560 (formerly patent application number 5348/DELNP/2014) dated 30/06/2014 in the name of 1. EMORY UNIVERSITY 2. GAVEGNANO, CHRISTINA 3. SCHINAZI, RAYMOND, F. titled "Pharmaceutical Compositions of JAK Inhibitors and Antiretroviral Agents for Treating or Preventing HIV" (amended title).

STATEMENT OF CASE OF OPPONENT

The Opponent herein respectfully submits as under:

1. A Post-grant opposition under Section 25(2) of the Patents Act, 1970, is being submitted by the Opponent against Indian Patent No. 427560 (hereinafter referred to as the "impugned patent") which has been granted in favour of 1. EMORY UNIVERSITY 2. GAVEGNANO, CHRISTINA 3. SCHINAZI, RAYMOND, F. (Hereinafter referred to as the "Applicant/Patentee").

2. The Opponent, Sankalp Rehabilitation Trust is society formed under the Societies Registration Act. The said trust is a community of dedicated individuals working to stop the spread of HIV and empower drug users to take charge of their health and lives. The trust believes that every person has a right to live and has been actively pursuing its goal of aiding patients afflicted by HIV/AIDS in all walks of their life including legal assistance. Sankalp has stood by drug users and attempted to change their lives and improve their health by procuring and supplying medicines for rehabilitation, running camps, advising drug users etc.

3. The trust has also been actively involved in championing the cause of patients affected by HIV-AIDS. For example in August 2008, the Ministry of Health and Family Welfare's NACO developed an "Office Memorandum" in response to a public interest litigation (PIL), in which the Supreme Court reviewed the steps taken by NACO to combat HIV/AIDS and the services being provided to patients. The Supreme Court had issued directives, including those aimed at improving the sufficiency of centres distributing second line anti-retroviral therapy (ART).

4. Subsequently, Sankalp Rehabilitation Trust & ANR filed a writ petition challenging the criteria employed by NACO for administering ART for persons suffering from HIV/AIDS, specifically the extension of second line treatment to all patients who need it, regardless of whether they underwent first line treatment in private or government sector, and regardless of whether that first line treatment was rational. NACO consistently expressed that its ultimate goal was to have universal second line treatment. In proceedings before the Supreme Court, the parties discussed the modalities to bring about

universal second line treatment to all those who need it. Thus, the Opponent trust has been taking up the cause of HIV-AIDS patients at all times.

5. The Opponent has an interest in filing the present post-grant opposition as it is affected by the continuance of the impugned patent on the register – would result in denial of access to important medicnes to HIV-AIDS patients. The Petitioner-Opponent believes that the patent is granted in error is accordingly filing the present post-grant opposition to oppose the patent.

6. The present opposition is against Indian patent no. 427560 which was originally filed as Indian Patent Application No. 5348/DELNP/2014 on 30/06/2014. The said application was granted on 29/03/2023 with patent number 427560 (hereinafter referred as the Impugned Patent). The Impugned patent is titled "PHARMACEUTICAL COMPOSITIONS OF JAK INHIBITORS AND ANTIRETROVIRAL AGENTS FOR TREATING OR PREVENTING HIV" (amended title). The priority date of the impugned patent is 30/11/2011. The impugned patent application was published in the official journal of the patent office on 31/03/2023. The Impugned Patent Application is the national phase application of PCT/US2012/067369 and draws its priority from US application 61/564,994 dated 30 Nov 2011, US application 61/570,813 dated 14 Dec 2011.

7. The Impugned Patent Application is entitled “Pharmaceutical Compositions of JAK Inhibitors and Antiretroviral Agents for Treating or Preventing HIV” (amended title)

8. The Opponent by way of this present post-grant opposition submits that the claims currently pending on record are not patentable under the provisions provided in this Act. The claims as filed and currently on record are annexed herewith as **Annexure-1** and reproduced herein below for ready reference:

1. A composition for treating or preventing HIV comprising:

a) an effective antiviral amount of a JAK inhibitor selected from the group consisting of CEP-701 (Lestaurtinib), AZD1480, LY3009104/INCB28050 Pacritinib/SB1518, VX-509, GLPG0634, INC424, R-348, CYT387, TG 10138, AEG 3482, 7-iodo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acryl amide, 7-(3-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phen- yl)acrylamide, N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, methyl 2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxylate, N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(4-amino-3-methoxyphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, N,N-dimethyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 1-ethyl-3-(2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)urea, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)metha- nesulfonamide, 2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)pheno- l, 2-cyano-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)phenyl)acetamide, N-(cyanomethyl)-2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxamide, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 1-ethyl-3-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)-2-(-trifluoromethoxy)phenyl)urea 20. N-(3-nitrophenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(3-nitrophenyl)thieno[3,2-d]pyrimidin-2-amine, N1-(7-(2-ethylphenyl)thieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N1-(7-iodothieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, 7-(4-amino-3-(tri fluoromethoxy)phenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl-)acetamide, N-(cyanomethyl)-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-

yl)phenyl)methanesulfonamide, N-(cyanomethyl)-N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(3-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)b- enzenesulfonamide, N-(4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-iodo-N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(2-isopropylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine 40. N7-(2-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, N7-(4-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, 7-(5-amino-2-methylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, 7-iodo-N-(3-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine,. 7-(4-amino-3-nitrophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-methoxypyridin-3-yl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(7-iodothieno[3,2-d]pyrimidin-2-ylamino)phenyl)methanol, N-tert-butyl-3-(2-(3-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N-tert-butyl-3-(2-(3-(hydroxymethyl)phenylamino)thieno[3,2-d]pyrimidin-7--yl)benzenesulfonamide, N-(4-morpholinophenyl)-7-(4-nitrophenylthio)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(4-amino-3-nitrophenyl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine 53. N-(3,4-dimethoxyphenyl)-7-(2-methoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4-dimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)b-enzenesulfonamide, 7-(2-aminopyrimidin-5-yl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,6-dimethoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,4-dimethoxypyrimidin-5-yl)thieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide,. 2-cyano-N-(4-methyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide,. ethyl 3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-

7-yl)benzoate, 7-bromo-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine,. N-(3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(cyanomethyl)-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)benzamide,. N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, tert-butyl-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-pyrazole-1-carboxylate, 7-bromo-N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-((4-ethylpiperazin-1-yl)methyl)phenylamino)thieno[3,- 2-d]pyrimidin-7-yl)benzenesulfonamide, N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]- pyrimidin-7-yl)benzenesulfonamide, tert-butyl pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, 3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(3-chloro-4-fluorophenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno- [3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl-)-1H-pyrazole-1-carboxylate, 7-(benzo[d][1,3]dioxol-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 5-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-ind- ole-1-carboxylate, 7-(2-aminopyrimidin-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-5,6-di-hydropyridine-1(2H)-carboxylate, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-phenyl)acetamide, N-(4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-(4-(4-methylpiperazin-1-yl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno- [3,2-d]pyrimidin-2-amine, N-(2-methoxy-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, 7-

bromo-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, N-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzyl)methanesulfonamide, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(4-(morpholinomethyl)phenyl)-7-(3-(piperazin-1-yl)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(6-(2-morpholinoethylamino)pyridin-3-yl)-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-(aminomethyl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(1-ethylpiperidin-4-yloxy)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(2,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, and pharmaceutically acceptable salts and prodrugs thereof, and

b) highly active antiretroviral therapy (HAART) agent comprising:

a) at least one of an adenine, cytosine, thymidine, and guanine nucleoside antiviral agent, or

b) at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors.

2. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is zidovudine (AZT).

3. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is 2R,4R)-2-amino-9-[(2-hydroxymethyl)-I, 3-dioxolan-4-yl]adenine (DAPD) or (−)-β-D-2-aminopurine dioxolane (APD).

4. The composition of Claim 1, wherein the antiretroviral agents comprise:

a) cis-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ((-)-FTC) or (−)-cis-2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (3TC),

b) 9-[(R)-2[[bis[[isopropoxycarbonyl)oxy]-methoxy]-phosphinyl] methoxy] propyl] adenine fumarate (TDF),

c) DAPD or APD,

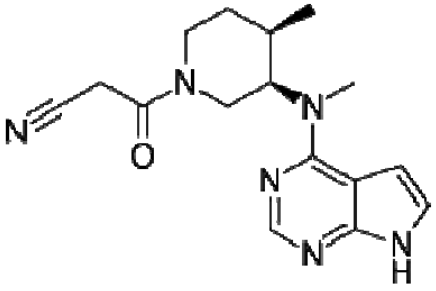
d) AZT, and

e) a NNRTI, a protease inhibitor, or an integrase inhibitor.

5. The composition of Claim 4, wherein the NNRTI is Sustiva, the protease inhibitor is Kaletra, or the integrase inhibitor is Raltegravir or Elvitegravir.

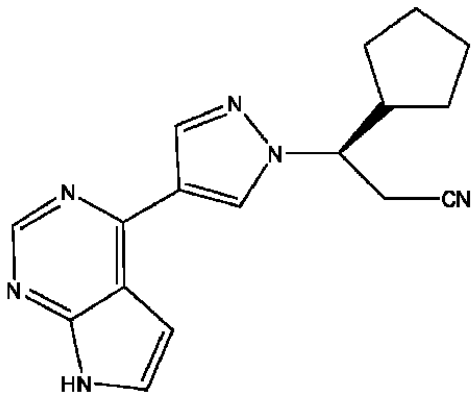
6. The composition of Claim 1, wherein the HAART agent(s) comprises at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors

7. The composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof.

8. The composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof. .

9. The composition of any of Claims 1-8, wherein the composition further comprises a macrophage depleting agent.

10. The composition of Claim 9, wherein the macrophage depleting agent is Boniva or Fosamax.

11. The composition of any of Claims 1-9, further comprising a reactivation agent.

12. The composition of Claim 11, wherein the reactivation agent is panobinostat.

9. **PRIOR ARTS:** The opponent wishes to rely on the following prior arts as evidence in support of the grounds of opposition.
   i. WO2011109217A2 (WO'217) published 09 September 2011 (**Annexed herewith as Annexure 2**)
   ii. Richman et al., "The Challenge of Finding a Cure for HIV Infection"; Science, vol 323, published 6 March 2009 (**Annexed herewith as Annexure 3**)
   iii. Margolis David M., "Mechanisms of HIV Latency: an Emerging Picture of Complexity"; Curr HIV/AIDS Rep (2010) 7:37–43 published 27 January 2010 (**Annexed herewith as Annexure 4**)
   iv. Gavegnano et al., "Antiretroviral therapy in macrophages: implication for HIV eradication"; Antiviral Chemistry & Chemotherapy 2009 20: 63–78 (**Annexed herewith as Annexure 5**)

10. It is submitted that the claims of impugned patent application are liable to be refused on following grounds as below, which are without prejudice to each other:
   (a) Section 25(2)(e): Lack of inventive step
   (b) Section 25(2)(f): Invention is not patentable under section 3(d) and 3(e)
   (c) Section 25(2)(g): The complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.
   (d) Section 25(2)(h): Failure to disclose the information required by section 8 of the Patents Act.

GROUND 3: Section 25(1)(e) Lack of Inventive Step

11. It is submitted that the invention as claimed is obvious and does not involve any inventive step in view of the disclosures published prior to the earliest priority date of the impugned patent application i.e. prior to 03/09/2020.

12. It is submitted that claims 1-30 of the impugned application lack inventive step and are obvious in view of common general knowledge in art and combined with teachings of the following-

    - WO2011109217A2 (WO'217) published 09 September 2011 (Annexure 2)
    - Richman et al., "The Challenge of Finding a Cure for HIV Infection"; Science, vol 323, published 6 March 2009 (Annexure 3)
    - Margolis David M., "Mechanisms of HIV Latency: an Emerging Picture of Complexity"; Curr HIV/AIDS Rep (2010) 7:37–43 published 27 January 2010 (Annexure 4)
    - Gavegnano et al., "Antiretroviral therapy in macrophages: implication for HIV eradication"; Antiviral Chemistry & Chemotherapy 2009 20: 63–78 (Annexure 5)

13. It is submitted that WO'217 relates to a method for inhibiting virus replication by administration of a composition including a JAK2 kinase inhibitor wherein said virus is dependent on RNA polymerase for replication (para 3, internal page 2).

14. It is disclosed (para 1, internal page 2) that by inhibiting JAK2 kinase and/**or** the phosphorylation of HEXIM1, viral replication can be inhibited, thereby providing a method of treating RNA polymerase dependent virus based disorders such as Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2). Thus, WO'217 contemplates inhibition of JAK2 kinase as an alternative option to phosphorylation of HEXIM1.

15. WO'217 discloses that the JAK2 kinase inhibitor can be administered as a composition (claim 1)

16. Thus, WO'217 discloses targeting of viruses that use RNA polymerase, especially HIV, by inhibition of janus kinase that can be administered as a composition.

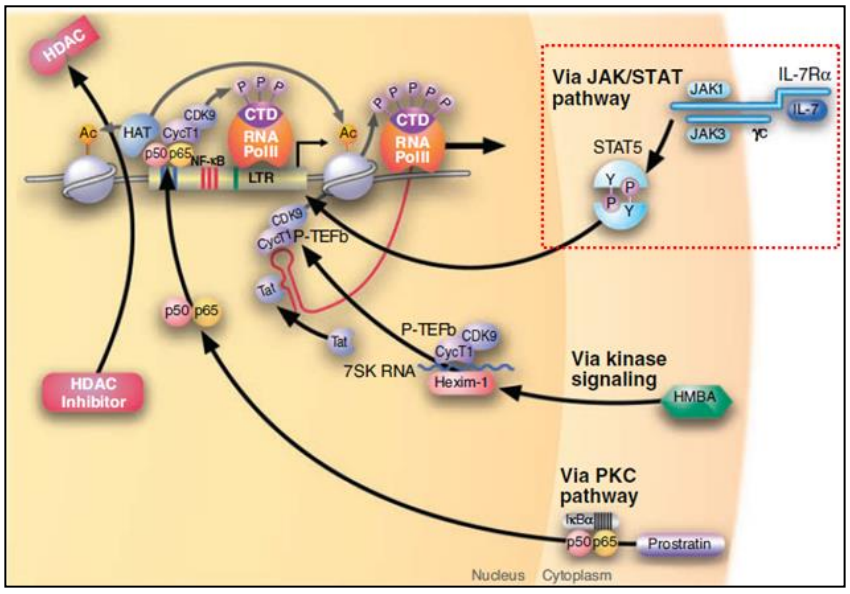
17. Richman et al. relates to the current challenges faced in achieving a complete eradication of HIV because of the inability of highly active antiretroviral therapy (HAART) to target the latent HIV existing in certain reservoirs in the body, especially $CD4^+$ T cells.

18. It is disclosed that HAART, even though successful, has limitations such as the necessity of treatment to be maintained for life (column 1, internal page 1304).

19. It is further disclosed that, despite the prolonged suppression of HIV replication below the standard limits of detection for patients on HAART, ongoing viremia can be detected at levels of 1 to 50 copies per milliliter in the majority of patients and that The suboptimal penetration of many antiretrovirals into the central nervous system may also permit low levels of viral replication and/or release from stable viral reservoirs (column 2, internal page 1304). It is further disclosed that Patient data reveal that 1 in 10^6 $CD4^+$ T cells are latently infected with HIV, despite the durable suppression of detectable plasma viremia (column 3, internal page 1304). A further mechanism complicating things is thought to be that in vivo, these cells are intermittently activated by antigen recognition or as bystanders in a local inflammatory process, which leads to the release of progeny virions.

20. The concept of HIV latency is discussed, (Table 1, internal page 1304) which highlights that latently infected resting memory $CD4^+$ T cells are the best characterized latent reservoir for HIV-1. The persistence of latently infected memory $CD4^+$ T lymphocytes precludes their elimination by HAART alone for the lifetime of the patient.

21. Richman further contemplates various strategies and pathways that can be targeted in order to disrupt latent proviral HIV infection, shown in Figure 2 (internal page 1306)



22. It is disclosed that Interleukin 7 (IL-7), a cytokine essential for maintenance of T cell homeostasis, can induce HIV expression from quiescent resting cells without global T

cell activation, via the JAK/STAT5 signaling pathway (Figure 2) Thus, with a goal of attempting to suppress HIV in such cells, administration of JAK inhibitors to inhibit the JAK/STAT5 signaling pathway is suggested, since this pathway activates HIV expression.

23. It is further disclosed that activation from latency to completion of the replication cycle should result in lytic cell death of CD4+ T cells. Multiple mechanisms may contribute to the maintenance of proviral latency and so**, combination approaches could be required to eradicate infection**. Such strategies would depend on current or future antiretroviral therapy to completely inhibit all new infection events (column 2 and 3, internal page 1305)

24. Further, Richman discusses potential strategies for purging persistent proviral infection and discloses that any inductive agent(s) used **must be utilized in combination with HAART to prevent further HIV spread to uninfected CD4$^+$ T cells** (Figure 3, internal page 1307).

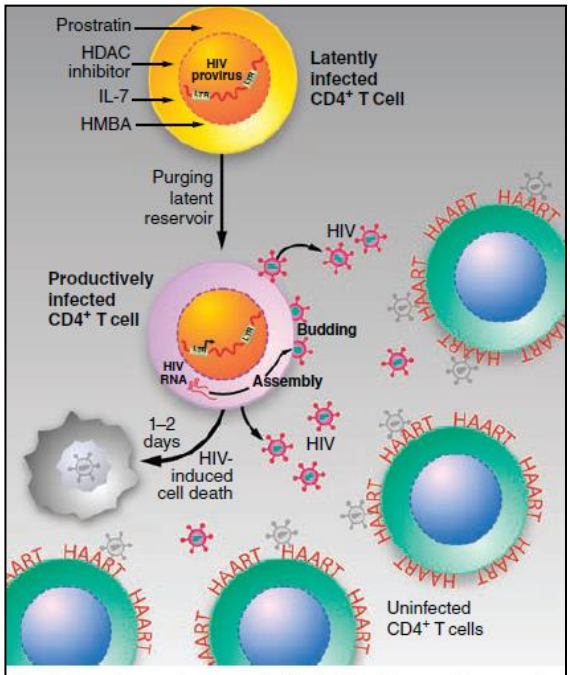


Fig. 3. Purging persistent proviral infection. If targeted approaches, alone or in combination, succeed in activating latent HIV proviruses present in differentiated CD4$^+$ T cells, the life span of these cells should be short. These inductive agents must be used in combination with HAART to prevent further HIV spread to uninfected CD4$^+$ T cells.

25. Thus, Richman provides clear motivation to target latent HIV in reservoirs via the JAK/STAT pathway and also to use combination of inductive agents with HAART to purge persistent proviral infection.

26. Richman thus discloses the various strategies (including combination of different strategies) that could be used to target HIV, both active and that present in latent reservoirs.

27. Margolis David M. discloses mechanisms of HIV latency, stating that Multiple molecular mechanisms appear to underlie the establishment and maintenance of persistent, latent HIV infection, most frequent in the resting central memory $CD4^+$ T cell (abstract, internal page 37).

28. The success as well as limitations of HAART in controlling HIV is discussed. Margolis further discloses that IL-7, a cytokine essential for maintenance of T-cell homeostasis, can induce HIV expression from quiescent resting cells without global T-cell activation, via the JAK/STAT5 signaling pathway (Conclusions, para 2, internal page 41).

29. Thus, it was known that the JAK/STAT5 signalling pathway is directly implicated in HIV expression from resting cells.

30. It is submitted that Gavegnano et al. discloses HIV infected macrophages as potential long-term HIV reservoir that must be selectively destroyed in order to achieve HIV eradication (Introduction, internal page 63).

31. Gavegnano discloses the severe neurological effects of chronic infection with HIV, as a result of a central nervous system (CNS) infiltration of the HIV virus.

32. The sequence of productive infection in macrophage reservoirs leading to repopulation of latently infected macrophage reservoirs, further leading to continual maintenance of macrophage/microglial latent viral reservoirs is discussed (Figure 1, internal page 64). It is disclosed that this leads to an inability to eradicate HIV infection.

33. Thus, Gavegnano et al. clearly discloses the macrophage as reservoir of latent HIV, and the direct role of such infected macrophages in bringing about neurological impairment

in HIV infected individuals. The need to target and eradicate this reservoir is clearly disclosed.

34. In summary, it can be seen that the prior art (Annexures 1-4) clearly lay out the motivation to arrive at the presently impugned application. WO'217 unambiguously discloses the targeting of viruses that use RNA polymerase (especially HIV) by using janus kinase 2 inhibitors. It was therefore known that inhibition of janus kinase could be used to taerget the HIV virus. Further, Richman et al. discloses the tendency of the HIV virus to resist eradication by hiding in certain reservoirs such as $CD4^+$ T cells, and that such reservoirs represent an impediment to the complete eradication of HIV by HAART therapy alone. Richman discloses that JAK/STAT pathway represents a potential target for latent HIV present in reservoirs and further that combination therapy with HAART would be highly desirable to be used with inductive agents. Margolis discloses latency of HIV in various reservoirs and that IL-7 can induce HIV expression from quiescent resting cells without global T-cell activation, via the JAK/STAT5 signalling pathway, thus pointing towards JAK/STAT5 as a clear target, given the goal of eliminating latent HIV. Finally, Gavegnano discloses that macrophages are also a reservoir for latent HIV and, when not eliminated, the severe congnitive and CNS decline as a result of HIV persisting in these cells.

35. Thus, Annexures 1-4 clearly teach that the motivation to utilize a combination of agent, one targeting janus kinase (for eliminating latent HIV in reservoirs) and one addressing other targets (HAART for active non-latent HIV in the body). The impugned patent claims composition with just such a combination.

36. Thus, the subject matter claimed in impugned patent application is obvious and lacks inventive step. In view of the above submissions, impugned application lacks inventive step and therefore, should be rejected on this ground alone.

Post filing data submitted with response to FER

37. The Applicant had submitted data entitled Annexures 1-4, along with response to the FER in support of its patent application. It is submitted that all this was generated and submitted several years after the priority date of the patent application, and as such there

is no link between the specification as filed, and the post-filing data, because none of the examples in the specification show the data of the actual composition claimed.

However, the Opponent is herein Addressing all the Annexures as follows:

Annexure I

38. Annexure I is a copy of a presentation referencing scientific journal articles published several years after priority date of the impugned patent. Internal pages 2-15 deal with the rationale underpinning the use of JAK inhibitors in anti-HIV therapy. As already elaborated, the role of JAK in downstream pathways implicated in HIV maintenance and proliferation in the body was already well known in the art, predating the priority date of the patent.

39. Pages 16-25 refer to an article by Gavegnano et al. in the Journal of Clinical Investigation, without annexing the actual article itself. From the contents of Annexure I only, it appears that said article shows the effect of ruxolitinib and tofacitinib on the reservoirs thought to harbour HIV (e.g. T cells). The changes in levels of several markers indicating lifespan of the HIV-harboring cells are outlined. It is stated that ruxolitinib and tofacitinib have effects like significantly reducing markers for reservoir lifespan in HIV reservoirs (page 18), block HIV replication in (page 20) and inhibit T-cell markers associated with viral persistence, increased reservoir size and reseeding (page 21), along with other effects indicating that these JAK inhibitors are able to target HIV reservoir cells.

40. It is submitted that, these effects are downstream of the JAK/STAT pathway. Targeting this pathway was already well known in the prior art as a viable solution to targeting HIV reservoirs cells where HIV persists despite the success of HAART therapy, as discussed previously.

41. Further, the effects outlined above refer only to that of the JAK inhibitors, not to effect of the composition actually claimed. Thus, there is indication if the composition as claimed is more efficacious than the single class of agent (JAK inhibitors). Further, no comparison to effect of standard HAART therapy is shown.

42. Further, the presentation refers to an unpublished work (Page 28) and to research by Gavegnano and Schinazi et al (AAC, 2013) relating to baricitinib anti-HIV properties in primary human cells. The parameters shown are antiviral potency, reservoir reseeding, reservoir size and lifespan and lastly, CNS trafficking and inflammation. Comparison with ruxolitinib and the antiviral drug 3TC (lamivudine) is shown. It can be seen that, with respect to antiviral potency, 3TC outperformed baricitinib in both PBM and macrophages ($EC_{50/90}$).

43. Further, like the previous data, the one shown above is only shown for single agent from a single class (baricitinib/ruxolitinib), not for the composition claimed, which is directed to agents from two separate classes (JAK+HAART).

44. The presentation refers to an article (page 30, Annexure I) titled "The Janus kinase inhibitor ruxolitinib reduces HIV replication in human macrophages and ameliorates HIV encephalitis in a murine model" by Haile et al. A study of this publication shows that the study done therein deals with assessing the impact of ruxolitinib for its potential to inhibit HIV-1 replication in macrophages and HIV-induced activation in monocytes/macrophages in culture. Also, the study deal with a murine model of HIV encephalitis (HIVE) was used to assess the impact of ruxolitinib on histopathological features of HIVE, brain viral load, as well as its ability to penetrate the blood-brain-barrier (BBB). It is disclosed (page 41) that ruxolitinib decreases in vivo markers of HIV-induced encephalitis in a murine CNS HIV model. It can be seen from the above that the entire study has been conducted on antiviral effect of ruxolitinib alone. Therefore, it does not deal with the antiviral effect of a composition of a JAK inhibitor and a HAART agent(s). This publication does not demonstrate that a composition (which is claimed in impugned patent) is more effective than a standard antiviral therapy (such as standard HAART therapy).

45. The presentation Annexure I further refers to ruxolitinib and HAART therapy regimen administered to monkeys (pages 31-34). The slide on page 32 shows how the ruxolitinib and HAART were administered from 0 to 6 weeks. It can be seen that the ruxolitinib is administered at the start of regimen (0 week). The HAART regimen is administered at approx. the 4 week mark. Thus, it is clear that the JAK inhibitor and HAART are <u>not</u> being administered together as part of a single composition, but separately.

46. Further, the results of administering a regimen of JAK inhibitor and HAART in monkeys is shown on page 33. The testing involved comparison of 2.1 mg/kg Jakafi twice daily, versus 2.1 mg/kg Jakafi + HAART (Kaletra + (-)-FTC + PMPA + Raltegravir, with no drug administration used as control. It can be seen that the comparison shown here is between a JAK inhibitor alone, versus a combination of JAK inhibitor and HAART. It is pertinent to point out here that the more accurate comparison would have been that between a combination of JAK inhibitor and HAART versus HAART therapy alone. In the absence of such a comparison, it is cannot be concluded from this result that the combination of JAK inhibitor and HAART therapy is more effective than standard HAART therapy alone.

47. Page 34 shows the levels of various markers following administration of ruxolitinib treatment on the monkey model. It is disclosed that 2 of 4 monkeys responded to ruxolitinib treatment. Thus, not only is this effect not that of a combined administration of JAK+HAART, it can be seen that even the effect of ruxolitinib alone is only 50% on the tested model.

Annexure 2

48. Annexure 2 relates to the numerous neurological complications following HIV infection in humans that manifest several years after the infection. The life-altering consequences of HIV associated neurocognitive disorder are discussed. Annexure 2 discloses that with the advent of HAART therapy HIV infected patients develop a less severe form of cognitive disorder (called minor cognitive motor disorder), instead of AIDS dementia complex (ADC), which patients used to develop before advent of HAART therapy. Thus, Annexure 2 underscores the important and significant impact of HAART on slowing and mitigating cognitive decline in HIV infected patients.

49. Therefore, any therapy/composition that supposedly also slows and mitigates neurocognitive decline in HIV infected individuals must demonstrate such effects above and beyond those obtained by HAART alone.

Annexure 3

50. Annexure 3 is a study relating to investigating effects of ruxolitinib and tofacitinib on an HIV reservoir ($CD4^+$ T cells). The effect of both ruxolitinib and tofacitinib (presumably representing all JAK inhibitors), on these cells was studied via certain parameters thought to be major impediments to full eradication of HIV. These parameters are inhibition of cytokine-induced STAT5 phosphorylation and Bcl-2 expression, inhibition of viral replication, reduction frequency of cells harboring integrated HIV viral DNA and block IL-15 induced reactivation of latent HIV, inhibition of HIV bystander infection.

51. It is submitted that, with respect to the effect of ruxolitinib and tofacitinib on the STAT5 phosphorylation, Annexure 3 initially states (internal page 3/30) that pSTAT5 expression remained significantly associated with integrated HIV DNA. To monitor STAT5 phosphorylation, in γ-C receptor cytokine stimulated total CD4 T cells and in the different memory subsets known to harbor the HIV reservoir in the presence or absence of the JAK inhibitors ruxolitinib and tofacitinib (internal page 5/30). It is shown that ruxolitinib reduced pSTAT5 expression, the same being shown for tofacitinib. However, it is not stated that the cells that were utilized in above test actually are infected cells. The above test is simply resting on the premise that the reduction of pSTAT5 expression in above cells (like CD4 T cells), and reduction of frequencies of such cells, indirectly shows that pSTAT5 reduction would also work in infected cells.

52. With respect to inhibition of HIV replication, (internal page 7/30 – 9/30), it is submitted that Annexure 3 shows reduced p24 production by CD4+ T cells isolated from viremic donors and stimulated with CD3/CD28 when compared to DMSO controls (internal page 7/30). The inhibition of production of HIV by ruxolitinib from infected cells is shown with both ART and without ART. It can be seen that the EC_{90} of ruxolitinib with ART is 6.2 μM, which is actually ***lower*** that of ruxolitinib alone (0.26 μM). ART in this context is presumably an acronym for antiretroviral therapy. This data therefore undermines the actual basis of using the claimed composition, which is a directed towards a combination of (a) JAK inhibitor and (b) HAART agent (which includes standard ART agents), if it is being shown that the antiviral potency of individual agent is more than that of the combination.

53. Further, the effect of ruxolitinib and tofacitinib on the maintenance of the existing reservoir was assessed (internal page 9/30 - 11/30). Cultures were generated in the

presence of, and in the absence of ART. It is disclosed that ruxolitinib and tofacitinib significantly ($p < 0.05$) decreased the frequency of cells with integrated DNA in cultures of T cells activated by TCR in the presence or absence of ART. It can be seen (Fig. 3A and 3B) that, the effect of using ruxolitinib and tofacitinib on CD4 cells (with integrated HIV DNA), with and without ART is nearly the same for ruxolitinib. There is negligible difference between the effect of administering ruxolitinib alone, or with ART, as measured by reduction of log copies per million CD4 cells. For tofacitinib, the effect of tofacitinib alone is actually better at reducing log copies per million CD4 cells than the tofacitinib + ART. It is submitted that both these observed effects undermine the invention that the patent has claimed (JAK inhibitor + HAART agent). This is because the effects demonstrated above (FIG. 3A & 3B) are for JAK + ART. The ART is antiretroviral therapy; therefore, ART can be included in HAART.

54. With respect to the ability of ruxolitinib to inhibit the infection of bystander cells i.e. uninfected CD4 T cells, Annexure 3 speculates, based on ruxolitinib treated cells to withstand infection, that Jak-STAT inhibitors could synergize with ART and decrease seeding of the HIV reservoir in uninfected bystander cells (last line, internal page, 11/30). However, no actual data is presented therein for this anticipated effect.

Annexure 4

55. Annexure 4 is a declaration (affidavit) by Raymond F. Schinazi and Christina Gavegnano that was submitted in connection with the corresponding EP application 12852554.0 at the EPO. The affidavit states (para 16, internal page 5) that reference D9 (cited at the EPO) disclosed using tofacitinib, which falls within the scope of formula A, which is no longer claimed in the application.

56. However, dependent claim 7 of the impugned patent still claims tofacitinib (or a pharmaceutically salt or prodrug thereof) despite having disclaimed it at the EPO, and despite having submitted the Schinazi et al. affidavit at the IPO which states this fact. The Applicant has not provided any reason for why, despite this declaration, the claim directed to tofacitinib was retained in the patent granted in India.

57. In addition to the above, it is submitted that much of the data submitted as annexures along with response to the FER includes tofacitinib in the examples that establish effect of JAK inhibitor activity against HIV, which the application disclaimed at the EPO.

58. Further, the affidavit states (pt. (4), internal page 7 and para 18, internal page 6), based on data in a paper referred to as soon-to-be published, that concentrations of JAK inhibitors required to block viral production are 2-5 fold higher when no antiviral is present to shut down the bulk of ongoing replication (i.e. when JAK inhibitors are not used in combination with HAART). It is submitted that fold change is not a true parameter of potency. Further, the paragraph referred to shows that ruxolitinib antiviral potency without ART is 0.007 μM; EC_{90} 0.26 μM and ruxolitinib antiviral potency with ART is EC_{50} 0.17 μM; EC_{90} 6.2 μM. The concentrations of ruxolitinib at which this occurs is shown to be 0.01, 0.1, 1.0 and 10 μM. Thus, it appears that at the same concentration of ruxolitinib, the potency of ruxolitinib + ART is actually lower than that of ruxolitinib itself.

GROUND 2: Claims not patentable under Section 25(1)(f)

The claimed subject matter in not patentable under Section 3(d) of the Act

59. It is submitted that the impugned patent application falls within the purview of section 3(d) of the Patents Act, 1970 which states that *"the mere discovery of a new form of a known substance which does not result in the enhancement of the known efficacy of that substance or the mere discovery of any new property or new use for a known substance or of the mere use of a known process, machine or apparatus unless such known process results in a new product or employs at least one new reactant.*

    Explanation -For the purposes of this clause, salts, esters, ethers, polymorphs, metabolites, pure form, particle size, isomers, mixtures of isomers, complexes, combinations and other derivatives of known substance shall be considered to be the same substance, unless they differ significantly in properties with regard to efficacy."

60. The Opponent submits that the composition claimed in present impugned patent is comprises of known JAK inhibitors and known highly active antiretroviral agents (HAART). Not a single compound (drug) in the list of both of these classes of agents is

new. The Applicant has contended (in the written submission pursuant to the section 14 hearing) the following:

"The Applicant asserts that when the claimed composition is not known in the art, there does not arise a question of applicability of Section 3(d) at the first place, and based on this reason alone, the objection over the invention claimed in claims 1-13 falling within the purview of Section 3(d) is not tenable in the eyes of law."

Here, Opponent humbly submits that, in the judgment delivered by the Hon'ble Delhi High Court in Best Agrolife Limited v Deputy Controller of Patents & Anr (WP (C)-IPD 11/2022 & CM 32/2022, 54/2022, 55/2022), the Court observed that-

"*Section 3(d) deals with assessment of 'enhanced efficacy' of claimed composition in comparison to efficacy of known substance and not merely comparison of efficacy with individual components.....*"

Thus, the Applicant's argument in this regard is fallacious.

61. Further, it is submitted that the 'enhanced efficacy' in a case such as this, where the invention seeks to achieve the goal of complete eradication of HIV (via the targeting of latent HIV in reservoir in addition to HIV in non-reservoir parts of the body), ought to have been established by showing <u>no rebound of HIV</u> some years after treatment. Such data would have shown that HIV in reservoirs has indeed been eliminated. In the absence of such data, there is no difference in standard HIV therapy and the composition of the present patent.

62. Further, in the post-filing annexures submitted by Applicant in response to FER, it can be seen that <u>none</u> of these actually show the effect of an <u>actual composition</u> against HIV.

63. Further, Annexure A, for example, shows data in which both classes of agents are not even being administered together, but sequentially, which undermines the entire premise of the invention (which claims a composition).

64. Therefore, the impugned application falls squarely within the purview of section 3(d) of the Patents Act 1970 and ought to be rejected.

<u>The claimed subject matter in not patentable under Section 3(e) of the Act</u>

65. It is submitted that the impugned patent application falls within the purview of section 3(e) of the Patents Act, 1970 which states that "*a substance obtained by a mere admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance*".

66. It is humbly submitted that claims of the impugned patent are directed to composition comprising (a) JAK inhibitor and (b) HAART agent. The Applicant has contended the following (in the written submission pursuant to the section 14 hearing) with regard to the objection under section 3(e)-

> **The Applicant has already put on record, numerous evidences including requisite experimental data (See Annexure 1-4, submitted along with reply to FER)** evincing the fact that inventors of the present invention surprisingly found that there exists a synergism between JAK inhibitors and HAART agents, wherein presence of JAK inhibitors in the composition significantly increased the efficacy of HIV treatments by performing roles not performed by HAART alone. Further, it was found that concentration of the JAK inhibitors required to block viral production is reduced by 2-5 fold lower when a direct-acting antiviral agent such as HAART(s) is present to shut down the viral replication, **which clearly illustrates the synergistic effect.**
>
> Attention of the Ld. Controller was drawn towards the experimental data provided as part of the **Annexure 3** (particularly, at internal Page 7 of 30) and Expert Declaration provided as **Annexure 4** (particularly, at internal Pages 5 and 6) that evinces the aforesaid facts.

67. The Opponent has addressed this data under the inventive step argument. Specifically, regarding the above data referenced by the Applicant, it is submitted that –

68. Firstly, the Opponent submits that the post-filed annexures (filed with FER response) demonstrates data on only two JAK inhibitors (ruxolitinib and tofacitinib), one of which is disclaimed at the EPO (tofacitinib). However, the Applicant has claimed a large number of JAK inhibitors in the claimed composition, based on just this data.

69. With respect to inhibition of HIV replication, (internal page 7/30 – 9/30), it is submitted that Annexure 3 shows reduced p24 production by CD4+ T cells isolated from viremic donors and stimulated with CD3/CD28 when compared to DMSO controls (internal page 7/30). The inhibition of production of HIV by ruxolitinib from infected cells is shown with both ART and without ART. It can be seen that the EC_{90} of ruxolitinib with ART is 6.2 µM, which is actually ***lower*** that of ruxolitinib alone (0.26 µM). ART in this context

is presumably an acronym for antiretroviral therapy. This data therefore undermines the actual basis of using the claimed composition, which is a directed towards a combination of (a) JAK inhibitor and (b) HAART agent (which includes standard ART agents), if it is being shown that the antiviral potency of individual agent is more than that of the combination.

70. Further, the effect of ruxolitinib and tofacitinib on the maintenance of the existing reservoir was assessed (internal page 9/30 - 11/30). Cultures were generated in the presence of, and in the absence of ART. It is disclosed that ruxolitinib and tofacitinib significantly ($p < 0.05$) decreased the frequency of cells with integrated DNA in cultures of T cells activated by TCR in the presence or absence of ART. It can be seen (Fig. 3A and 3B) that, the effect of using ruxolitinib and tofacitinib on CD4 cells (with integrated HIV DNA), with and without ART is nearly the same for ruxolitinib. There is negligible difference between the effect of administering ruxolitinib alone, or with ART, as measured by reduction of log copies per million CD4 cells. For tofacitinib, the effect of tofacitinib alone is actually better at reducing log copies per million CD4 cells than the tofacitinib + ART. It is submitted that both these observed effects undermine the invention that the patent has claimed (JAK inhibitor + HAART agent). This is because the effects demonstrated above (FIG. 3A & 3B) are for JAK + ART. The ART is antiretroviral therapy; therefore, ART can be included in HAART.

71. With respect to the ability of ruxolitinib to inhibit the infection of bystander cells i.e. uninfected CD4 T cells, Annexure 3 speculates, based on ruxolitinib treated cells to withstand infection, that Jak-STAT inhibitors could synergize with ART and decrease seeding of the HIV reservoir in uninfected bystander cells (last line, internal page, 11/30). However, no actual data is presented therein for this anticipated effect. Thus, no synergistic effect has been demonstrated.

72. Thus the impugned application falls within the purview of section 3(e) of the Patents Act, 1970 and should be refused on the said ground.

The claimed subject matter in not patentable under Section 3(i) of the Act

73. It is submitted that the impugned patent application falls within the purview of section 3(i) of the Patents Act, 1970 which states that "*any process for the medicinal, surgical, curative, prophylactic [diagnostic, therapeutic] or other treatment of human beings or any process for a similar treatment of animals to render them free of disease or to increase their economic value or that of their products.*"

74. The Opponent humbly submits that the claims of the patent are directed towards a composition comprising two separate classes of agents (a) JAK inhibitor and (b) HAART agent (s).

75. However, while the HAART agent(s) were already known to be active against HIV, the JAK inhibitor(s) are compounds that were also already known to target the JAK/STAT pathway.

76. The only facet that was not known is the purported activity of JAK inhibitors against HIV. Therefore, the Applicant has merely discovered a new use for these already known JAK inhibitor(s).

77. Thus, it is humbly submitted that the above claims fall within the purview of section 3(i) of the Patents Act, 1970 and should be refused on the said ground.

GROUND 3: INSUFFICIENCY OF DISCLOSURE [section 25(1)(g)]

78. It is submitted that complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.

79. It is submitted that it is a well settled law that the specification should clearly and fairly describe the invention and disclose the best mode of working the invention so that the person skilled in the art could perform the invention without any undue efforts. Further, it is submitted that claims of impugned application are not fairly based on the specification and the complete specification does not fairly describe the invention and the method by which it is to be performed.

80. Claim 1 (as well as dependent claims 2-12) is directed to composition for treating or preventing HIV comprising (a) a JAK inhibitor and (b) a HAART agent, where the HAART agent comprises at least one agent that could be from different classes of

antiviral agents (a) an (b). However, the specification of the impugned patent does not include even a single example of any type of composition with any active agent(s).

81. The Applicant, in its written submission to the section 14 hearing (page 6) has referenced the following (Lines 23-33 on page 95) in the complete specification as Disclosure of exemplary formulations comprising JAK inhibitor and HAART agent in the as-filed specification:

> *In one embodiment,* ***the composition is a co-formulated pill, tablet, or other oral drug delivery vehicle including one or more of the JAK inhibitors described herein, and optionally including one or more additional antiviral agents.***
>
> *In another embodiment,* ***the JAK inhibitors described herein are co-formulated with ATRIPLA®*** *(efavirenz 600 mg/emtricitabine [(-)-FTC] 200 mg/tenofovir disoproxil fumarate 300 mg), and,* ***optionally, with a thymidine nRTI such as AZT and a guanine nRTI (or a compound such as DAPD which is deaminated in vivo to 30 form a guanine nRTI, in this case, DXG****). Because efavirenz is an NNRTI, tenofovir is an adenine nRTI, (-)-FTC is a cytosine nRTI, and AZT is a thymidine nRTI, and DAPD is deaminated in vivo to form DXG (a guanine NRTI),* ***the combination of the coformulated compounds will provide, in addition to the JAK inhibitors…..***

However, the claims are much broader than what is described above. Further, in the description referred to above, the proportion of the JAK inhibitor is not specified. Therefore, even if a person skilled in the art was to consider the teaching above, the missing teaching with respect to proportion of JAK inhibitor would render the effort to make the claimed composition extremely difficult.

82. The claims are directed to a composition comprising a JAK inhibitor and a HAART agent, where the HAART agent comprises at least one agent incorporated from separate and distinct classes of antiviral agents. However, no ratio of the JAK inhibitor to that of the HAART agent(s) incorporated in the composition is specified in the claims. Thus, there is no indication of how much of each active agent is to be incorporated in the composition claimed.

83. The claims specify a large number of JAK inhibitors that can be incorporated in the composition of invention. Further, the composition, as per the claim, can incorporate any combination of JAK inhibitor(s) and HAART agent(s) specified in claims, so this necessarily means that the claims cover a very large number of permutations and combinations of these active agents. However, none of the examples in the specification is directed to the actual composition comprising both the JAK inhibitor and HAART agent(s). The activity data demonstrated in the specification is only for establishing the activity of JAK inhibitor(s), not the composition actually claimed. Since the Applicant has claimed a composition comprising more than one class of active agents, it is incumbent upon the Applicant to establish the anti-HIV activity of the composition itself.

84. Further, the Annexures 1-4, as submitted by the Applicant along with response to the FER, also do not include examples of the composition claimed in the granted claims.

85. The claims 1, 7, 8 claim the 'prodrug' form of the JAK inhibitors therein, apart from the specific JAK inhibitors themselves. It is submitted that derivatives which are suitable for use as 'prodrugs' vary in terms of their structure from one active molecule to another. Moreover, each species is likely to be different in respect of how any given molecule is absorbed, metabolised, enters the plasma etc. so that the skilled person is put under an undue burden to determine whether any given molecule is an effective 'prodrug' in any given species. Furthermore, the expression 'prodrug' is unclear in respect of the maximum and minimum pharmacokinetic parameters (for example, C_{max}, area under the curve, bioavailability, rate and extent of metabolism. Therefore, the claims directed to the 'prodrug' of JAK inhibitors lack support in the specification as well as annexures 1-4 submitted by the Applicant.

86. Thus, the specification does not sufficiently and clearly describe the invention or the method by which it is to be performed and is liable to be rejected on said ground.

GROUND 5: INFORMATION RELATING TO CORRESPONDING APPLICATIONS UNDER SECTION 8 [SECTION 25(1)(H)]

87. The Applicant has failed to disclose to the Patent Office the information required under Section 8.The Applicant is required to provide all the information regarding the

prosecution of the equivalent applications till the grant of the Indian application to the Patent Office in writing from time to time and also within the prescribed time.

88. It is observed that Applicant has not provided information about updated the status of corresponding application in the Form-3 which information has not been provided to the learned Controller.

89. Therefore, the applicant has failed to comply with the requirements of the section 8 of the act and the opponent demands rejection on this ground also.

90. It is submitted that the Applicant has failed to disclose the details of corresponding foreign applications and impugned patent application to be refused.

91. The opponents crave leave to file further submissions and evidence with respect to this ground.

CONCLUSION

92. In view of the above, the claims are not inventive and not patentable and insufficient. The post-grant opposition as filed may be allowed and the subject patent application may be refused.

HEARING REQUESTED

93. The Opponent hereby requests a hearing under section 25(2) of the Patents Act, 1970 (hereinafter referred to as "the Patents Act") and Rule 55 of the Patents Rules (hereinafter referred to as "the Rules").

P R A Y E R

In the fact and circumstances of the case, the Opponent prays as follows:

i. that the Controller take the present Opposition on record; that the Indian Patent number 427560 (formerly patent application number 5348/DELNP/2014), be rejected under Section 25(2) of the Patents (Amendment) Act, 2005;

ii. that the Opponent may be allowed to file further documents and evidence if necessary to support their averments;

iii. that the Opponent may be allowed to file rejoinder and affidavit if necessary to support their averments;

iv. that the Opponent may be granted an opportunity of being heard in the matter before any final orders are passed;

v. that the Opponent may be allowed to make further submissions in case the Patentee makes any amendments in the claims;

vi. any other reliefs considering the facts and circumstances may be granted in favour of the Opponent in the interest of justice.

Dated this 21st day of December, 2023

PRAGYA SINGH THAKUR (IN /PA – 3329)
OF RAJESHWARI AND ASSOCIATES
AGENT FOR THE OPPONENT

TO,
THE CONTROLLER OF PATENTS
THE PATENT OFFICE, NEW DELHI

AMENDED CLAIMS: MARKED COPY

We Claim:

1. A ~~pharmaceutical~~ composition for treating or preventing HIV comprising ~~a combination of~~:

a) an effective antiviral amount of a JAK inhibitor selected from the group consisting of CEP-701 (Lestaurtinib), AZD1480, LY3009104/INCB28050 Pacritinib/SB1518, VX-509, GLPG0634, INC424, R-348, CYT387, TG 10138, AEG 3482, 7-iodo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acryl amide, 7-(3-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phen- yl)acrylamide, N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, methyl 2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxylate, N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(4-amino-3-methoxyphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, N,N-dimethyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 1-ethyl-3-(2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)urea, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)metha- nesulfonamide, 2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)pheno- l, 2-cyano-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)phenyl)acetamide, N-(cyanomethyl)-2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxamide, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 1-ethyl-3-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)-2-(-trifluoromethoxy)phenyl)urea 20. N-(3-nitrophenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(3-nitrophenyl)thieno[3,2-d]pyrimidin-2-amine, N1-(7-(2-ethylphenyl)thieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N1-(7-iodothieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, 7-(4-amino-3-(tri fluoromethoxy)phenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl-)acetamide, N-

(cyanomethyl)-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(cyanomethyl)-N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(3-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-y-l)phenyl)methanesulfonamide, 4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)b- enzenesulfonamide, N-(4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-iodo-N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(2-isopropylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine 40. N7-(2-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, N7-(4-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, 7-(5-amino-2-methylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, 7-iodo-N-(3-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine,. 7-(4-amino-3-nitrophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-methoxypyridin-3-yl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(7-iodothieno[3,2-d]pyrimidin-2-ylamino)phenyl)methanol, N-tert-butyl-3-(2-(3-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N-tert-butyl-3-(2-(3-(hydroxymethyl)phenylamino)thieno[3,2-d]pyrimidin-7--yl)benzenesulfonamide, N-(4-morpholinophenyl)-7-(4-nitrophenylthio)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(4-amino-3-nitrophenyl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine 53. N-(3,4-dimethoxyphenyl)-7-(2-methoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4-dimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)b-enzenesulfonamide, 7-(2-aminopyrimidin-5-yl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,6-dimethoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,4-dimethoxypyrimidin-5-yl)thieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide,. 2-cyano-N-(4-methyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-

yl)phenyl)acetamide,. ethyl 3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzoate, 7-bromo-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine,. N-(3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(cyanomethyl)-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)benzamide,. N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, tert-butyl-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-pyrazole-1-carboxylate, 7-bromo-N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-((4-ethylpiperazin-1-yl)methyl)phenylamino)thieno[3,- 2-d]pyrimidin-7-yl)benzenesulfonamide, N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]- pyrimidin-7-yl)benzenesulfonamide, tert-butyl pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, 3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(3-chloro-4-fluorophenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno- [3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl-)-1H-pyrazole-1-carboxylate, 7-(benzo[d][1,3]dioxol-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 5-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-ind- ole-1-carboxylate, 7-(2-aminopyrimidin-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-5,6-di-hydropyridine-1(2H)-carboxylate, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-phenyl)acetamide, N-(4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-(4-(4-methylpiperazin-1-yl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno- [3,2-d]pyrimidin-2-amine, N-(2-methoxy-4-(2-(4-

(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, 7-bromo-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, N-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzyl)methanesulfonamide, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(4-(morpholinomethyl)phenyl)-7-(3-(piperazin-1-yl)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(6-(2-morpholinoethylamino)pyridin-3-yl)-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-(aminomethyl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(1-ethylpiperidin-4-yloxy)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(2,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, and pharmaceutically acceptable salts and prodrugs thereof, and

b) highly active antiretroviral therapy (HAART) agent~~(s)~~. comprising:

a) at least one of an adenine, cytosine, thymidine, and guanine nucleoside antiviral agent, or

b) at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors.

~~2. The pharmaceutical composition of Claim 1, wherein the HAART agent(s) comprising:~~

~~a) at least one each of an adenine, cytosine, thymidine, and guanine nucleoside antiviral agent, or~~

~~b) at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors.~~

~~3~~2. The ~~pharmaceutical~~ composition of Claim ~~2~~1, wherein the thymidine nucleoside antiretroviral agent is zidovudine (AZT).

~~4~~3. The ~~pharmaceutical~~ composition of Claim ~~2~~1, wherein the thymidine nucleoside antiretroviral agent is 2R,4R)-2-amino-9-[(2-hydroxymethyl)-I, 3-dioxolan-4-yl]adenine (DAPD) or (−)-β-D-2-aminopurine dioxolane (APD).

~~5~~4. The ~~pharmaceutical~~ composition of Claim ~~2~~1, wherein the antiretroviral agents comprise:

a) cis-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ((-)-FTC) or (−)-cis-2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (3TC),

b) 9-[(R)-2[[bis[[isopropoxycarbonyl)oxy]-methoxy]-phosphinyl] methoxy] propyl] adenine fumarate (TDF),

c) DAPD or APD,

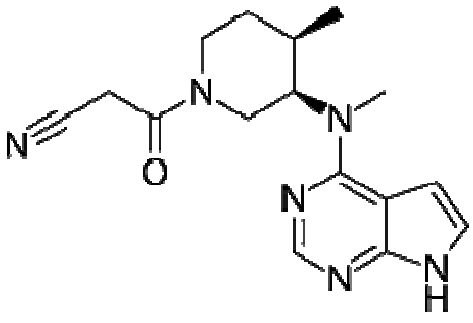
d) AZT, and

e) a NNRTI, a protease inhibitor, or an integrase inhibitor.

~~6~~5. The ~~pharmaceutical~~ composition of Claim ~~5~~4, wherein the NNRTI is Sustiva, the protease inhibitor is Kaletra, or the integrase inhibitor is Raltegravir or Elvitegravir.

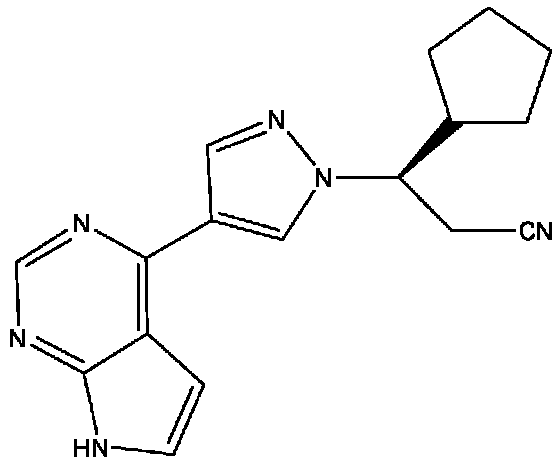
~~7~~6. The ~~pharmaceutical~~ composition of Claim ~~2~~1, wherein the HAART agent(s) comprises at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors

~~8~~7. The ~~pharmaceutical~~ composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof.

~~9~~8. The ~~pharmaceutical~~ composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof. .

~~10~~9. The ~~pharmaceutical~~ composition of any of Claims 1-~~9~~8, wherein the composition further comprises a macrophage depleting agent.

~~11~~10. The ~~pharmaceutical~~ composition of Claim ~~10~~9, wherein the macrophage depleting agent is Boniva or Fosamax.

~~12~~11. The ~~pharmaceutical~~ composition of any of Claims 1-~~10~~9, further comprising a reactivation agent.

~~13~~12. The ~~pharmaceutical~~ composition of Claim ~~12~~11, wherein the reactivation agent is panobinostat.

AMENDED CLAIMS: CLEAN COPY

We Claim:

1. A composition for treating or preventing HIV comprising:

a) an effective antiviral amount of a JAK inhibitor selected from the group consisting of CEP-701 (Lestaurtinib), AZD1480, LY3009104/INCB28050 Pacritinib/SB1518, VX-509, GLPG0634, INC424, R-348, CYT387, TG 10138, AEG 3482, 7-iodo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acryl amide, 7-(3-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phen- yl)acrylamide, N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, methyl 2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxylate, N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(4-amino-3-methoxyphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, N,N-dimethyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 1-ethyl-3-(2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)urea, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)metha- nesulfonamide, 2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)pheno- l, 2-cyano-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)phenyl)acetamide, N-(cyanomethyl)-2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxamide, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 1-ethyl-3-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)-2-(-trifluoromethoxy)phenyl)urea 20. N-(3-nitrophenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(3-nitrophenyl)thieno[3,2-d]pyrimidin-2-amine, N1-(7-(2-ethylphenyl)thieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N1-(7-iodothieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, 7-(4-amino-3-(tri fluoromethoxy)phenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl-)acetamide, N-(cyanomethyl)-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-

yl)phenyl)methanesulfonamide, N-(cyanomethyl)-N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(3-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-y-l)phenyl)methanesulfonamide, 4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)b- enzenesulfonamide, N-(4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-iodo-N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(2-isopropylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine 40. N7-(2-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, N7-(4-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, 7-(5-amino-2-methylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, 7-iodo-N-(3-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine,. 7-(4-amino-3-nitrophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-methoxypyridin-3-yl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(7-iodothieno[3,2-d]pyrimidin-2-ylamino)phenyl)methanol, N-tert-butyl-3-(2-(3-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N-tert-butyl-3-(2-(3-(hydroxymethyl)phenylamino)thieno[3,2-d]pyrimidin-7--yl)benzenesulfonamide, N-(4-morpholinophenyl)-7-(4-nitrophenylthio)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(4-amino-3-nitrophenyl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine 53. N-(3,4-dimethoxyphenyl)-7-(2-methoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4-dimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)b-enzenesulfonamide, 7-(2-aminopyrimidin-5-yl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,6-dimethoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,4-dimethoxypyrimidin-5-yl)thieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide,. 2-cyano-N-(4-methyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide,. ethyl 3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-

7-yl)benzoate, 7-bromo-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine,. N-(3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(cyanomethyl)-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)benzamide,. N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, tert-butyl-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-pyrazole-1-carboxylate, 7-bromo-N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-((4-ethylpiperazin-1-yl)methyl)phenylamino)thieno[3,- 2-d]pyrimidin-7-yl)benzenesulfonamide, N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]- pyrimidin-7-yl)benzenesulfonamide, tert-butyl pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, 3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(3-chloro-4-fluorophenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno- [3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl-)-1H-pyrazole-1-carboxylate, 7-(benzo[d][1,3]dioxol-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 5-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-ind- ole-1-carboxylate, 7-(2-aminopyrimidin-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-5,6-di-hydropyridine-1(2H)-carboxylate, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-phenyl)acetamide, N-(4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-(4-(4-methylpiperazin-1-yl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno- [3,2-d]pyrimidin-2-amine, N-(2-methoxy-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, 7-

bromo-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, N-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzyl)methanesulfonamide, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(4-(morpholinomethyl)phenyl)-7-(3-(piperazin-1-yl)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(6-(2-morpholinoethylamino)pyridin-3-yl)-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-(aminomethyl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(1-ethylpiperidin-4-yloxy)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(2,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, and pharmaceutically acceptable salts and prodrugs thereof, and

b) highly active antiretroviral therapy (HAART) agent comprising:

a) at least one of an adenine, cytosine, thymidine, and guanine nucleoside antiviral agent, or

b) at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors.

2. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is zidovudine (AZT).

3. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is 2R,4R)-2-amino-9-[(2-hydroxymethyl)-I, 3-dioxolan-4-yl]adenine (DAPD) or (−)-β-D-2-aminopurine dioxolane (APD).

4. The composition of Claim 1, wherein the antiretroviral agents comprise:

a) cis-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ((-)-FTC) or (−)-cis-2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (3TC),

b) 9-[(R)-2[[bis[[isopropoxycarbonyl)oxy]-methoxy]-phosphinyl] methoxy] propyl] adenine fumarate (TDF),

c) DAPD or APD,

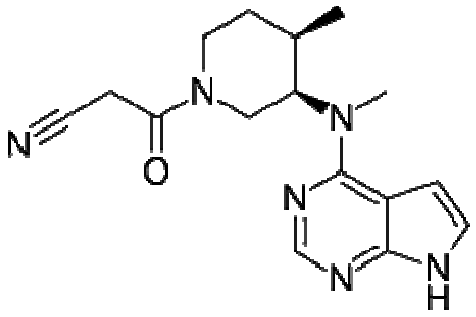
d) AZT, and

e) a NNRTI, a protease inhibitor, or an integrase inhibitor.

5. The composition of Claim 4, wherein the NNRTI is Sustiva, the protease inhibitor is Kaletra, or the integrase inhibitor is Raltegravir or Elvitegravir.

6. The composition of Claim 1, wherein the HAART agent(s) comprises at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors

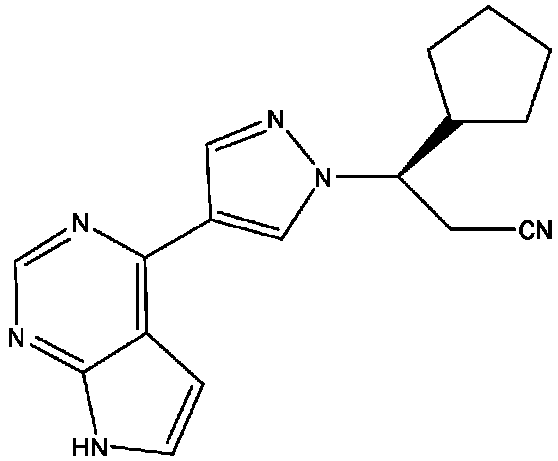
7. The composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof.

8. The composition of Claim 1, wherein the JAK inhibitor compound is

AMENDED CLAIMS: CLEAN COPY



, or a pharmaceutically acceptable salt or prodrug thereof. .

9. The composition of any of Claims 1-8, wherein the composition further comprises a macrophage depleting agent.

10. The composition of Claim 9, wherein the macrophage depleting agent is Boniva or Fosamax.

11. The composition of any of Claims 1-9, further comprising a reactivation agent.

12. The composition of Claim 11, wherein the reactivation agent is panobinostat.

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(54) **Title**: METHODS OF TREATING OR PREVENTING RNA POLYMERASE DEPENDENT VIRAL DISORDERS BY ADMINISTRATION OF JAK2 KINASE INHIBITORS

(57) **Abstract**: The present invention is directed to methods of treating or preventing RNA polymerase dependent viral disorders using JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation. In a particular aspect the present invention is directed to the treatment of prevention of HIV infection in a subject by administration of JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation.

METHODS OF TREATING OR PREVENTING RNA POLYMERASE DEPENDENT VIRAL DISORDERS BY ADMINISTRATION OF JAK2 KINASE INHIBITORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 61/309646, filed March 2, 2010, entitled Methods of Treating or Preventing RNA Polymerase Dependent Viral Disorders By Administration of JAK2 Kinase Inhibitors, which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention is directed to the use of methods of treating or preventing viral disorders using JAK2 kinase inhibitors and agents that inhibit HEXIM1 tyrosine phosphorylation.

BACKGROUND OF INVENTION

While human immunodeficiency virus (HIV) infection, which results in AIDS, is a relatively new infection in the human population, it has quickly risen to the foremost health problem in the world. HIV/AIDS is now the leading cause of death in sub-Saharan Africa, and is the fourth biggest killer worldwide. At the end of 2001, it was estimated that 40 million people were living with HIV infection worldwide. The Centers for Disease Control (CDC) estimates that nearly 800,000 people are living with AIDS in the U.S, and 40,000 new cases diagnosed each year. While better treatment methods are now known to prolong the life of patients with HIV infection, there is still no cure.

Modern anti-HIV drugs target several different stages of the HIV life cycle, and several of the enzymes that HIV requires to replicate and survive. Some of the commonly used anti-HIV drugs include nucleoside reverse transcriptase inhibitors such as AZT, ddI, ddC, d4T, 3TC, and abacavir; nucleotide reverse transcriptase inhibitors such as tenofovir; non-nucleoside reverse transcriptase inhibitors such as nevirapine, efavirenz and delavirdine; protease inhibitors such as saquinavir, ritonavir, indinavir, nelfinavir, amprinavir, lopinavir and atazanavir; and fusion inhibitors such as enfuvirtide.

SUMMARY OF INVENTION

The present invention is based, at least in part, on the elucidation of the role of JAK2 kinase and the tyrosine phosphorylation of HEXIM1 in the replication of RNA polymerase dependent viruses. Indeed, the present invention is predicated, in part, on the finding that JAK2 kinase plays an important role in phosphorylating HEXIM1, and that upon such phosphorylation, RNA polymerase II is activated so as to promote viral gene expression and viral replication after infection of a cell. Accordingly, by inhibiting JAK2 kinase and/or the phosphorylation of HEXIM1, the inventors have discovered that viral replication can be inhibited, thereby providing a method of treating RNA polymerase dependent virus based disorders such as Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2), Influenza (Influenzavirus A such as the H1N1 virus, Influenzavirus B and Influenzavirus C), Hepatitis (Hepatitis virus A, Hepatitis virus C, Hepatitis virus D and Hepatitis virus E), and the West Nile Virus.

Accordingly, the present invention provides a method for treating or preventing a viral disorder in a subject having or at risk of having said viral disorder, wherein said virus is RNA polymerase dependent, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby treating or preventing the viral disorder in said subject. In various embodiments, the viral disorder is selected from the group consisting of HIV, influenzavirus, influenzavirus A, influenzavirus B, influenzavirus C, H1N1, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus.

In another aspect, the present invention provides a method for inhibiting the replication of a virus in a subject, by administering to the subject infected with said virus, wherein said virus is dependent on RNA polymerase for replication, an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby inhibiting viral replication in the subject. In various embodiments, the virus is selected from the group consisting of HIV, influenzavirus, influenzavirus A, influenzavirus B, influenzavirus C, H1N1, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus.

In certain embodiments of the foregoing methods, the RNA polymerase is RNA polymerase II.

In another aspect, the invention is directed to a method for treating or preventing HIV infection in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby treating or preventing HIV in said subject. In yet another aspect, the invention is directed to a method for inhibiting the

replication of HIV in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby inhibiting HIV replication in the subject.

In certain embodiments, the method includes administering to the subject an effective amount of a JAK2 kinase inhibitor, for example, one that inhibits tyrosine kinase activity. In various embodiments, the JAK2 inhibitor is selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one, dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, or a combination thereof. In particular embodiments, the JAK2 kinase inhibitor is AG490 or 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one. In alternative embodiments, the JAK2 kinase inhibitor is selected from the group consisting of low molecular weight inhibitors, antibodies or antibody fragments, peptide or RNA aptamers, antisense constructs, small inhibitory RNAs and ribozymes. In a particular embodiment, the JAK2 kinase inhibitor is selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

In certain embodiments, the methods include administering to the subject an effective amount of an agent that inhibits tyrosine phosphorylation of HEXIM1. In a particular embodiment, the agent inhibits tyrosine phosphorylation of HEXIM1 at the YLEL domain of HEXIM1.

In a particular embodiment, the JAK2 kinase inhibitor or the agent that inhibits tyrosine phosphorylation of HEXIM1 is administered orally.

In further embodiments, the methods include monitoring the effectiveness of treatment, for example, monitoring the activity of JAK2 kinase in the subject or monitoring the phosphorylation of HEXIM1 at the YLEL domain.

In another aspect, the methods of the present invention include the treatment or prevention of HIV infection in a subject having or at risk of HIV infection, by administering to the subject an effective amount of an agent that inhibits phosphorylation of HEXIM1, for example, an agent that inhibits JAK2 mediated phosphorylation of HEXIM1 and/or tyrosine phosphorylation of HEXIM1 at the YLEL motif of HEXIM1, thereby treating or preventing HIV in said subject. In another aspect, the invention is directed to a method for treating or preventing HIV infection in a subject having or at risk of HIV

infection, by administering to the subject an effective amount of an agent that inhibits the release of HEXIM1 from a cyclin T1-cdk9 complex, thereby treating or preventing HIV in said subject.

For example, the agent that inhibits tyrosine phosphorylation of HEXIM1 may be selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one, dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline. In particular embodiments, the agent that inhibits tyrosine phosphorylation of HEXIM1 is a tyrphostin, such as AG490, or 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one. Alternatively, the agent is an antisense nucleic acid molecule selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

In a particular embodiment, the agent is administered orally.

In yet another aspect, the invention is directed to a method for identifying a therapeutic for treatment of HIV, by exposing a candidate substance to a composition including a JAK2 kinase or HEXIM1, monitoring the activity of the JAK2 kinase or HEXIM1, and identifying the candidate substance as a therapeutic for treatment of HIV if the candidate substance reduces the activity of the JAK2 kinase or the phosphorylation of HEXIM1. In a particular embodiment, the method involves monitoring the tyrosine phosphorylation of HEXIM1, wherein a reduction in the phosphorylation of HEXIM1 identifies the candidate substance as a therapeutic for treatment of HIV, and/or monitoring the JAK2 mediated phosphorylation of HEXIM1 at the YLEL motif of HEXIM1, wherein a reduction in the phosphorylation of HEXIM1 at the YXXL motif identifies the candidate substance as a therapeutic for treatment of HIV. Alternatively, or in addition, the method includes monitoring the release of HEXIM1 from a cyclin T1-cdk9 complex.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1A and Fig. 1B depict the results of the HIV-1 p24 antigen determination by Lateral Flow p24 antigen test on day 22 after exposure to Z3: Left to right, H9 cells (C), cells with inhibitor (Z3), Cells infected with HIV-1 (D-nef) and HIV-1 + Z3 (D-nef,Z3). The projected Z3 inhibition of endogenous HIV replication: > 90%.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, at least in part, on the elucidation of the role of JAK2 kinase and the tyrosine phosphorylation of HEXIM1 in the replication of RNA polymerase dependent viruses. Indeed, the present invention is predicated, in part, on the finding that JAK2 kinase plays an important role in phosphorylating HEXIM1, and that upon such phosphorylation, RNA polymerase II is activated so as to promote viral gene expression and viral replication after infection of a cell. Accordingly, by inhibiting JAK2 kinase and/or the tyrosine phosphorylation of HEXIM1, the inventors have discovered that viral replication can be inhibited, thereby providing a method of treating RNA polymerase dependent virus based disorders including, but not limited to lentivirus based disorders, such as Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2), Influenza (Influenzavirus A such as the H1N1 virus, Influenzavirus B and Influenzavirus C), Hepatitis (Hepatitis virus A, Hepatitis virus C, Hepatitis virus D and Hepatitis virus E), and the West Nile Virus.

Without wishing to be bound to any particular theory, the present invention is predicated upon the finding that JAK2 kinase serves to phosphorylate HEXIM1, specifically, at the YLEL epitope present on HEXIM1, so as to trigger the release of HEXIM1 from the transcription elongation factor P-TEFb complex (cyclin T1-cdk9). Upon release of HEXIM1 from the complex, a viral replicating factor, for example, TAT (an HIV replicating factor), complexes with the P-TEFb complex thereby hijacking the host cell's transcriptional machinery for purposes of promoting viral replication. Specifically, upon the interaction of the viral replicating factor with the P-TEFb complex, the P-TEFb complex, particularly, cdk9, triggers serine phosphorylation and transcription elongation of RNA Polymerase II so as to allow for transcription and replication of the viral genome.

Accordingly, based on this elucidated mechanism of action and, in particular, the JAK2 kinase phosphorylation of HEXIM1 at the YLEL motif, the inventors have identified that RNA polymerase dependent virus based disorders, such as Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2), Influenza (Influenzavirus A such as H1N1, Influenzavirus B and Influenzavirus C), Hepatitis (Hepatitis virus A, Hepatitis virus C, Hepatitis virus D and Hepatitis virus E), and the West Nile Virus, can be treated by use of JAK2 kinase inhibitors or agents that inhibit the tyrosine phosphorylation of HEXIM1.

Accordingly, in one aspect, the present invention is directed to a method for treating or preventing a viral disorder in a subject having or at risk of having said viral disorder, wherein said virus is RNA polymerase dependent, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits the tyrosine phosphorylation of HEXIM1, thereby treating or preventing the viral disorder (for example, HIV, influenzavirus, influenzavirus A such as H1N1, influenzavirus

B, influenzavirus C, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus) in said subject. In another aspect, the present invention is directed to a method for inhibiting the replication of a virus in a subject, by administering to the subject infected with said virus (for example, HIV, influenzavirus, influenzavirus A such as H1N1, influenzavirus B, influenzavirus C, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus), wherein said virus is dependent on RNA polymerase for replication, an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits the tyrosine phosphorylation of HEXIM1, thereby inhibiting viral replication in the subject.

In another aspect, the present invention is directed to a method for treating or preventing HIV infection in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits the tyrosine phosphorylation of HEXIM1, thereby treating or preventing HIV in said subject. In yet another aspect, the present invention includes methods for inhibiting the replication of HIV in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits the tyrosine phosphorylation of HEXIM1, thereby inhibiting HIV replication in the subject.

As used herein, "RNA polymerase" refers to the art recognized enzyme that assists in cellular production of RNA during transcription. Specifically, RNA polymerase catalyzes the initiation and elongation of each type of RNA, *i.e.,* mRNA, tRNA and rRNA, from a DNA template. In a particular embodiment, RNA polymerase refers specifically to RNA polymerase II, also referred to as RNA Pol II.

With respect specifically to viruses, RNA polymerases often serve an important function in viral genome replication and/or viral genome transcription, depending on the nature of the virus. For example, DNA viruses often utilize host cell DNA dependent RNA polymerases for transcription of viral DNA, thereby producing viral mRNAs.

RNA viruses use RNA polymerases to effect viral genome transcription and replication. For example, plus (+) strand RNA viruses possess RNA genomes that serve as mRNAs. While these viruses do not require RNA polymerase to initiate viral genome expression given that the RNA genome serves as the mRNA, viral encoded RNA dependent RNA polymerase is required to produce (-) strand RNA which in turn allows for production of more (+) strand RNA. These (+) strand RNA can serve as mRNAs, templates to make more (-) strand RNA or the genomes of progeny viruses.

Alternatively, minus (-) strand RNA viruses possess a viral genome which does not serve as mRNA. Accordingly, such viruses require the use of a viral RNA polymerase to transcribe the minus (-) strand RNA viruses to form (+) strands.

With respect specifically to HIV, RNA polymerase II serves to catalyze the replication of HIV genomic material and, further, to generate viral mRNA utilized to generate HIV proteins during translation. Specifically, upon HIV infection of a cell, the single stranded HIV RNA is converted to double-stranded HIV DNA which subsequently enters the host cell's nucleus and integrates within the host cell's DNA. The integrated viral DNA is referred to as a provirus. Upon activation of the host cell, the host cell's endogenous RNA polymerase is hijacked by the provirus so as to catalyze the replication of HIV genomic material and, further, to generate viral mRNA. Specifically, TAT, an HIV encoded transcription factor, binds to the TAT-Responsive element (TAR) in the viral RNA, prompting CDK9 to phosphorylate RNA polymerase II, which in turn serves to continue replication and elongation of the viral RNA.

As used herein, an "RNA polymerase dependent virus" refers to a virus that depends on RNA polymerase, for example, RNA Polymerase II, for expression or replication of the viral genome or spread of the virus. As well known in the art, RNA polymerase dependent viruses include certain RNA viruses that require RNA polymerase for viral genome replication such as, but not limited to, Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2), Influenzaviruses (Influenzavirus A such as H1N1, Influenzavirus B and Influenzavirus C), Hepatitis viruses (Hepatitis virus A, Hepatitis virus C, Hepatitis virus D and Hepatitis virus E), and the West Nile Virus.

As used herein, the terms "Janus Kinase 2" or " JAK2" or "JAK2 kinase" refer to the art recognized Janus kinase 2 protein that is involved in signaling of certain pathways, for example, the type II cytokine receptor pathway, the GM-CSF pathway and the gp130 receptor pathway. JAK2 is encoded by the JAK2 gene. The NCBI accession number for the JAK2 protein is AAY22962.

As used herein, the terms "JAK2 kinase inhibitor" or "JAK2 inhibitor" refer to any JAK2 kinase inhibitor that is currently known in the art or that will be identified in the future, and includes any chemical entity that, upon administration to a subject or cell, results in inhibition of a biological activity associated with the activity of JAK2 kinase in the cell or in the patient, including any of the downstream biological effects otherwise resulting from the activity of JAK2. Such JAK2 kinase inhibitors include any agent that can block JAK2 mediated phosphorylation or any of the downstream biological effects of JAK2 mediated phosphorylation that are relevant to treating viral disorders in a patient. Such an inhibitor can act by binding directly to ATP so as to prevent the phosphorylation of JAK2, thereby inhibiting its subsequent kinase activity.

In various embodiments of the present invention, JAK2 kinase inhibitors include, but are not limited to, low molecular weight inhibitors, antibodies or antibody fragments, peptide or RNA aptamers, antisense constructs, small inhibitory RNAs (*i.e.,* RNA

interference by dsRNA, RNAi) and ribozymes. In particular embodiments, the JAK2 kinase inhibitor is selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

In specific embodiments, JAK2 kinase inhibitors can be selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3), dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline. In a particular embodiment, the JAK2 kinase inhibitor is a tyrphostin such as AG490. In another embodiment, the JAK2 kinase inhibitor is the small molecule Z3.

As used herein, the term "HEXIM1" refers to the art recognized human hexamethylene bis-acetamide inducible 1 (HEXIM1) protein, also known as MAQ-1. HEXIM1 interacts with the pTEFb complex in a manner that activates RNA Polymerase II. The NCBI accession number for the HEXIM1 protein is BAA36166.

Further terms are defined herein so that the invention may be more readily understood.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used herein, the term "modulated" with respect to JAK2 kinase includes changing the expression, activity and/or function of JAK2 kinase in such a manner that it differs from the naturally-occurring expression, function and/or activity of JAK2 kinase under the same conditions. For example, the expression, function and/or activity can be greater or less than that of naturally occurring JAK2 kinase, *e.g.*, owing to a change in kinase activity, *etc*. As used herein, the various forms of the term "modulate" include stimulation (*e.g.*, increasing or upregulating a particular response or activity) and inhibition (*e.g.*, decreasing or downregulating a particular response or activity).

As used herein, the term "compound" includes any agent, *e.g.,* nucleic acid molecules, antisense nucleic acid molecule, peptide, peptidomimetic, small molecule, or other drug, which binds to JAK2 or has a stimulatory or inhibitory effect on, for example, JAK2 expression or activity, kinase affinity or stability. In one embodiment, the compound may modulate transcription of JAK2 or kinase activity of JAK2.

The term "inhibitor" or "inhibitory agent" includes agents which decrease the expression and/or activity of JAK2 or, alternatively, agents which inhibit the tyrosine

phosphorylation of HEXIM at, for example, the YLEL epitope. Exemplary inhibitory agents include, but are not limited to active protein and nucleic acid molecules, peptides and peptidomimetics of JAK2. Inhibitory agents also include naturally occurring inhibitors.

The agents of the invention can directly or indirectly modulate, *e.g.*, inhibit, the expression and/or activity of JAK2. Exemplary agents are described herein or can be identified using screening assays that select for such compounds, as described in detail below.

For screening assays of the invention, preferably, the "test compound" or "agent" screened includes molecules that are not known in the art to modulate, *e.g.*, inhibit, JAK2 activity and/or expression as described herein. Preferably, a plurality of agents are tested using the instant methods.

The term "library of test compounds" is intended to refer to a panel comprising a multiplicity of test compounds.

In one embodiment, the agent or test compound is a compound that directly interacts with JAK2 or directly interacts with a molecule with which JAK2 interacts (*e.g.*, a compound that inhibits the interaction between JAK2 and a JAK2 target molecule, *e.g.*, HEXIM1). In another embodiment, the compound is one that indirectly modulates, *e.g.*, inhibits, JAK2 expression and/or activity, *e.g.*, by inhibiting the activity of a molecule that is upstream or downstream of JAK2 in a signal transduction pathway involving JAK2. By way of example, the agent or test compound can serve to inhibit the phosphorylation of JAK2 by interacting with ATP, thereby preventing subsequent JAK2 mediated phosphorylation of downstream proteins such as HEXIM1. Such compounds can be identified using screening assays that select for such compounds, as described in detail below.

As used herein, the term "target molecule" or "binding partner" is a molecule with which JAK2 binds or interacts in nature, and which interaction results in a biological response. The target molecule can be a protein or a nucleic acid molecule. Exemplary target molecules of the invention include proteins in the same signaling pathway as the JAK2 protein, *e.g.*, proteins which may function upstream (including both stimulators and inhibitors of activity) or downstream of the JAK2 protein in a pathway involving for example, phosphorylation of JAK2 protein, phosphorylation of HEXIM1, release of HEXIM1 for the pTEFb complex, modulation of pTEFb complex activity, modulation of TAT interaction with RNA polymerase, modulation of CDK9 mediated phosphorylation of RNA polymerase, modulation of RNA polymerase activity, modulation of the transcription

of the viral genome, modulation of the replication of the viral genome, and modulation of the spread of a virus.

The term "interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a yeast two hybrid assay or coimmunoprecipitation. The term interact is also meant to include "binding" interactions between molecules. Interactions may be protein-protein or protein-nucleic acid in nature.

As used herein, the term "contacting" (*i.e.*, contacting a cell *e.g.* infected with a virus such as HIV, with a compound) is intended to include incubating the compound and the cell together *in vitro* (*e.g.,* adding the compound to cells in culture) or administering the compound to a subject such that the compound and cells of the subject are contacted *in vivo*.

As used herein, the term "indicator composition" refers to a composition that includes a protein of interest (*e.g.*, JAK2 kinase), for example, a cell that naturally expresses the protein, a cell that has been engineered to express the protein by introducing an expression vector encoding the protein into the cell, or a cell free composition that contains the protein (*e.g.*, purified naturally-occurring protein or recombinantly-engineered protein).

As used herein, the term "cell free composition" refers to an isolated composition which does not contain intact cells. Examples of cell free compositions include cell extracts and compositions containing isolated proteins.

As used herein an “agonist” of the JAK2 proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a JAK2 protein. An “antagonist” of a JAK2 protein can inhibit one or more of the activities of the naturally occurring form of the JAK2 protein by, for example, competitively modulating a cellular activity of a JAK2 protein.

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.,* complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

The term “small molecule” is a term of the art and includes molecules that are less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (*e.g.,* polyketides)

(Cane, *et al.* 1998. *Science* 282:63), and natural product extract libraries. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic.

As used herein, the term "peptide" includes relatively short chains of amino acids linked by peptide bonds. The term "peptidomimetic" includes compounds containing non-peptidic structural elements that are capable of mimicking or antagonizing peptides.

The term "treatment," as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, disorder, or infection, a symptom of a disease, disorder, or infection or a predisposition toward a disease, disorder, or infection, with the purpose of curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving or affecting the disease, disorder, or infection, the symptoms of disease, disorder, or infection or the predisposition toward a disease, disorder, or infection. A therapeutic agent includes, but is not limited to, nucleic acid molecules, small molecules, peptides, peptidomimetics, antibodies, ribozymes, and sense and antisense oligonucleotides described herein.

Various aspects of the invention are described in further detail in the following subsections:

I. JAK2 kinase inhibitors and Agents that Inhibit Tyrosine Phosphorylation of HEXIM1

In various aspects, the present invention is directed to JAK2 kinase inhibitors and agents that inhibit tyrosine phosphorylation of HEXIM1, and their use in therapeutic and prophylactic methods for the treatment of RNA polymerase dependent viruses. In various embodiments, the JAK2 kinase inhibitor or agent inhibiting HEXIM1 phosphorylation may be low molecular weight inhibitors, antibodies or antibody fragments, peptide or RNA aptamers, antisense constructs, small inhibitory RNAs (*i.e.,* RNA interference by dsRNA, RNAi), and ribozymes. For example, the JAK2 kinase inhibitor or agent inhibiting HEXIM1 tyrosine phosphorylation is selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA. Exemplary JAK2 kinase inhibitors or agents inhibiting HEXIM1 tyrosine phosphorylation further include such inhibitors identified by the methods described herein.

In certain embodiments, JAK2 kinase inhibitors or agents inhibiting HEXIM1 tyrosine phosphorylation can be selected from the group consisting of tyrphostin, AG 490 ((E)-2-Cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-pr openamide), lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, 2-methyl-1-phenyl-4-pyridin-2-yl-

2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3), dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline. In a particular embodiment, the JAK2 kinase inhibitor or agent inhibiting HEXIM1 tyrosine phosphorylation is a tyrphostin such as AG490. In another embodiment, the JAK2 kinase inhibitor or agent inhibiting HEXIM1 tyrosine phosphorylation is the small molecule Z3.

Small Molecules

In another embodiment, an inhibitory agent of the invention is a small molecule which interacts with JAK2 to thereby inhibit the activity of the JAK2 kinase or the tyrosine phosphorylation of HEXIM1. In another embodiment, the inhibitory agent of the invention is a small molecule which interacts with HEXIM1 to thereby inhibit the tyrosine phosphorylation of HEXIM1. Small molecule inhibitors of JAK2 or HEXIM1 tyrosine phosphorylation can be identified using database searching programs capable of scanning a database of small molecules of known three-dimensional structure for candidates which fit into the target protein site known in the art. Suitable software programs include, for example, CATALYST (Molecular Simulations Inc., San Diego, CA), UNITY (Tripos Inc., St Louis, MO), FLEXX (Rarey et al., *J. Mol. Biol*. 261: 470-489 (1996)), CHEM-3DBS (Oxford Molecular Group, Oxford, UK), DOCK (Kuntz *et al.*, *J. Mol. Biol* 161: 269-288 (1982)), and MACCS-3D (MDL Information Systems Inc., San Leandro, CA).

The molecules found in the search may not necessarily be leads themselves, however, such candidates might act as the framework for further design, providing molecular skeletons to which appropriate atomic replacements can be made. The scaffold, functional groups, linkers and/or monomers may be changed to maximize the electrostatic, hydrogen bonding, and hydrophobic interactions with the target protein. Goodford (Goodford *J Med Chem* 28:849-857 (1985)) has produced a computer program, GRID, which seeks to determine regions of high affinity for different chemical groups (termed probes) on the molecular surface of the binding site. GRID hence provides a tool for suggesting modifications to known ligands that might enhance binding. A range of factors, including electrostatic interactions, hydrogen bonding, hydrophobic interactions, desolvation effects, conformational strain or mobility, chelation and cooperative interaction and motions of ligand and enzyme, all influence the binding effect and should be taken into account in attempts to design small molecule inhibitors.

Small molecule inhibitors of JAK2 kinase activity or HEXIM1 tyrosine phosphorylation can also be identified using computer-assisted molecular design methods

comprising searching for fragments which fit into a binding region subsite and link to a predefined scaffold can be used. The scaffold itself may be identified in such a manner. Programs suitable for the searching of such functional groups and monomers include LUDI (Boehm, *J Comp. Aid. Mol. Des.* 6:61-78 (1992)), CAVEAT (Bartlett *et al.* in "Molecular Recognition in Chemical and Biological Problems", special publication of *The Royal Chem. Soc.*, 78:182-196 (1989)) and MCSS (Miranker *et al. Proteins* 11: 29-34 (1991)).

Yet another computer-assisted molecular design method for identifying JAK2 or HEXIM1 phosphorylation small molecule inhibitors comprises the *de novo* synthesis of potential inhibitors by algorithmic connection of small molecular fragments that will exhibit the desired structural and electrostatic complementarity with the active binding or phosphorylation site of the JAK2 or HEXIM1 protein. The methodology employs a large template set of small molecules with are iteratively pieced together in a model of the JAK2 or HEXIM1 binding or phosphorylation site. Programs suitable for this task include GROW (Moon *et al. Proteins* 11:314-328 (1991)) and SPROUT (Gillet *et al. J Comp. Aid. Mol. Des.* 7:127 (1993)).

The suitability of small molecule inhibitor candidates can be determined using an empirical scoring function, which can rank the binding affinities for a set of inhibitors. For an example of such a method see Muegge *et al.* and references therein (Muegge *et al.*, *J Med. Chem.* 42:791-804 (1999)). Other modeling techniques can be used in accordance with this invention, for example, those described by Cohen *et al.* (*J. Med. Chem.* 33: 883-894 (1994)); Navia *et al.* (*Current Opinions in Structural Biology* 2: 202-210 (1992)); Baldwin *et al.* (*J. Med. Chem.* 32: 2510-2513 (1989)); Appelt *et al.* (*J. Med. Chem.* 34: 1925-1934 (1991)); and Ealick *et al.* (*Proc. Nat. Acad. Sci. USA* 88: 11540-11544 (1991)).

Exemplary small molecule inhibitors for use in the present invention include, but are not limited to, tyrphostin, AG 490 ((E)-2-Cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-pr openamide), lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3), dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline. In a particular embodiment, the small molecule inhibitor is a tyrphostin such as AG490. In another embodiment, the small molecule inhibitor is Z3.

Antisense Molecules

An inhibitory agent of the invention can be, for example, an antisense nucleic acid molecule that is complementary to a gene encoding a JAK2 or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a particular polypeptide in a cell is well known in the art (see *e.g.*, Weintraub, H. *et al.* , Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986; Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule comprises a nucleotide sequence that is complementary to the coding strand of another nucleic acid molecule (*e.g.*, an mRNA sequence) and accordingly is capable of hydrogen bonding to the coding strand of the other nucleic acid molecule. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the coding region and an untranslated region (*e.g.*, at the junction of the 5' untranslated region and the coding region). Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element.

In another embodiment, an antisense nucleic acid of the invention is a compound that mediates RNAi. RNA interfering agents include, but are not limited to, nucleic acid molecules including RNA molecules which are homologous to the target gene or genomic sequence, *e.g.,* a JAK2 family member, or a fragment thereof, "short interfering RNA" (siRNA), "short hairpin" or "small hairpin RNA" (shRNA), and small molecules which interfere with or inhibit expression of a target gene by RNA interference (RNAi). RNA interference is a post-transcriptional, targeted gene-silencing technique that uses double-stranded RNA (dsRNA) to degrade messenger RNA (mRNA) containing the same sequence as the dsRNA (Sharp, P.A. and Zamore, P.D. 287, 2431-2432 (2000); Zamore, P.D., *et al. Cell* 101, 25-33 (2000). Tuschl, T. *et al. Genes Dev.* 13, 3191-3197 (1999)). The process occurs when an endogenous ribonuclease cleaves the longer dsRNA into shorter, 21- or 22-nucleotide-long RNAs, termed small interfering RNAs or siRNAs. The smaller RNA segments then mediate the degradation of the target mRNA. Kits for synthesis of RNAi are commercially available from, *e.g.* New England Biolabs and Ambion.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell. Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in the

culture medium *in vitro* or in the circulatory system or in interstitial fluid *in vivo*. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species.

Given the coding strand sequences encoding JAK2 disclosed herein (*see* Figure 1), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of JAK2 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of JAK2 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of JAK2 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length.

Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated region of an mRNA. An antisense nucleic acid for inhibiting the expression of a JAK2 polypeptide in a cell can be designed based upon the nucleotide sequence encoding the a JAK2 polypeptide, constructed according to the rules of Watson and Crick base pairing.

An antisense nucleic acid can exist in a variety of different forms. For example, the antisense nucleic acid can be an oligonucleotide that is complementary to only a portion of a JAK2 gene. To inhibit the expression of a JAK2 in cells in culture, one or more antisense oligonucleotides can be added to cells in culture media, typically at about 200 μg oligonucleotide/ml.

An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-

isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which direct constitutive, tissue specific or inducible expression of antisense RNA. For example, for inducible expression of antisense RNA, an inducible eukaryotic regulatory system, such as the Tet system (*e.g.*, as described in Gossen, M. and Bujard, H. (1992)

Proc. Natl. Acad. Sci. USA 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313) can be used. The antisense expression vector is prepared as described above for recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus. The antisense expression vector is introduced into cells using a standard transfection technique, as described above for recombinant expression vectors.

Given the coding strand sequence encoding JAK2 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of a JAK2 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of a JAK2 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of a JAK2 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.,* an antisense oligonucleotide) can be chemically synthesized using naturally occurring

nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.,* phosphorothioate derivatives and acridine substituted nucleotides may be used. Examples of modified nucleotides which may be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a JAK2 protein to thereby inhibit expression of the protein, *e.g.,* by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.,* by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier, *et al.* (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue, *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue, *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave JAK2 mRNA transcripts to thereby inhibit translation of JAK2 mRNA. A ribozyme having specificity for a JAK2 -encoding nucleic acid can be designed based upon the nucleotide sequence of a JAK2 cDNA disclosed herein (*see* Figure 1). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a JAK2 -encoding mRNA. See, *e.g.*, Cech, *et al.* U.S. Patent No. 4,987,071; and Cech, *et al.* U.S. Patent No. 5,116,742. Alternatively, JAK2 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, JAK2 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the JAK2 (*e.g.*, the JAK2 promoter and/or enhancers) to form triple helical structures that prevent transcription of the JAK2 gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

In yet another embodiment, the JAK2 nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to serve as antisense or antigene agents. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup, B. *et al.* (1996) *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase

peptide synthesis protocols as described in Hyrup, B. *et al.* (1996) *supra*; Perry-O'Keefe, *et al. Proc. Natl. Acad. Sci., USA* 93: 14670-675.

Such PNAs of JAK2 nucleic acid molecules can be used in therapeutic applications as described herein. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication.

Anti-JAK2 Antibodies

An isolated JAK2 polypeptide, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind JAK2 using standard techniques for polyclonal and monoclonal antibody preparation. A full-length Jak2 polypeptide can be used or, alternatively, the invention provides antigenic peptide fragments of JAK2 for use as immunogens. The antigenic peptide of JAK2 comprises at least 8 amino acid residues of the amino acid sequence as shown in Figure 1 and encompasses an epitope of JAK2 such that an antibody raised against the peptide forms a specific immune complex with JAK2. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Preferred epitopes encompassed by the antigenic peptide are regions of JAK2 that are located on the surface of the polypeptide, *e.g.*, hydrophilic regions, as well as regions with high antigenicity.

A JAK2 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed JAK2 polypeptide or a chemically synthesized JAK2 polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic JAK2 preparation induces a polyclonal anti- JAK2 antibody response.

Accordingly, another aspect of the invention pertains to anti- JAK2 antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as JAK2. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind JAK2. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein,

refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of JAK2. A monoclonal antibody composition thus typically displays a single binding affinity for a particular JAK2 polypeptide with which it immunoreacts.

Polyclonal anti-JAK2 antibodies can be prepared as described above by immunizing a suitable subject with a JAK2 immunogen or a nucleic acid molecule encoding the same. The anti- JAK2 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized JAK2. If desired, the antibody molecules directed against JAK2 can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti- JAK2 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown, *et al.* (1981) *J. Immunol.* 127:539-46; Brown, *et al.* (1980) *J. Biol. Chem* .255:4980-83; Yeh, *et al.* (1976) *Proc. Natl. Acad. Sci., USA* 76:2927-31; and Yeh, *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor, *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole, *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.,* 54:387-402; M. L. Gefter, *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a JAK2 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds JAK2.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti- JAK2 monoclonal antibody (see, *e.g.*, G. Galfre, *et al.* (1977) *Nature* 266:55052; Gefter, *et al. Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the

present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind JAK2, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti- JAK2 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with JAK2 to thereby isolate immunoglobulin library members that bind JAK2. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner, *et al.* U.S. Patent No. 5,223,409; Kang, *et al.* PCT International Publication No. WO 92/18619; Dower, *et al.* PCT International Publication No. WO 91/17271; Winter, *et al.* PCT International Publication WO 92/20791; Markland, *et al.* PCT International Publication No. WO 92/15679; Breitling, *et al.* PCT International Publication WO 93/01288; McCafferty, *et al.* PCT International Publication No. WO 92/01047; Garrard, *et al.* PCT International Publication No. WO 92/09690; Ladner, *et al.* PCT International Publication No. WO 90/02809; Fuchs, *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay, *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse, *et al.* (1989) *Science* 246:1275-1281; Griffiths, *et al.* (1993) *EMBO J.* 12:725-734; Hawkins, *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson, *et al.* (1991) *Nature* 352:624-628; Gram, *et al.* (1992) *Proc. Natl. Acad. Sci., USA* 89:3576-3580; Garrad, *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom, *et al.* (1991) *Nuc. Acids Res.* 19:4133-4137; Barbas, *et al.* (1991) *Proc. Natl. Acad. Sci., USA* 88:7978-7982; and McCafferty, *et al.* *Nature* (1990) 348:552-554.

Additionally, recombinant anti- JAK2 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention.

Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson, *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger, *et al.* PCT International Publication No. WO 86/01533; Cabilly, *et al.* U.S. Patent No. 4,816,567; Cabilly, *et al.* European Patent Application 125,023; Better, *et al.* (1988) *Science* 240:1041-1043; Liu, *et al.* (1987) *Proc. Natl. Acad. Sci., USA* 84:3439-3443; Liu, *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun, *et al.* (1987) *Proc. Natl. Acad. Sci., USA* 84:214-218; Nishimura, *et al.* (1987) *Canc. Res.* 47:999-1005; Wood, *et al.* (1985) *Nature* 314:446-449; and Shaw, *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi, *et al.* (1986) *BioTechniques* 4:214; Winter, U.S. Patent 5,225,539; Jones, *et al.* (1986) *Nature* 321:552-525; Verhoeyan, *et al.* (1988) *Science* 239:1534; and Beidler, *et al.* (1988) *J. Immunol.* 141:4053-4060. In one embodiment, an anti- JAK2 antibody is a fully human antibody.

Dominant Negative Inhibitors

Yet another form of an inhibitory agent of the invention is an inhibitory form of human JAK2, also referred to herein as a dominant negative inhibitor. Many proteins are known to homodimerize and to heterodimerize. One means to inhibit the activity of molecules that form dimers is through the use of a dominant negative inhibitor that has the ability to dimerize with a functional molecule but that lacks the ability to perform its normal biological activity (see e.g., Petrak, D. et al. (1994) J. Immunol. 153:2046-2051). By dimerizing with JAK2, such dominant negative inhibitors can inhibit their functional activity.

Accordingly, an inhibitory agent of the invention can be a form of a JAK2 polypeptide that has the ability to dimerize with other proteins but that lacks the ability to perform its normal biological activity, such as phosphorylation of HEXIM1. This dominant negative form of a JAK2 polypeptide may be, for example, a mutated form of JAK2 in which the conformational structure of the JAK2 has been altered to prevent phosphorylation of JAK2. Such dominant negative human JAK2 proteins can be expressed in cells using a recombinant expression vector encoding the JAK2 polypeptide, which is introduced into the cell by standard transfection methods. To express a mutant form of JAK2 lacking residues critical for phosphorylation, for example, at the YXXL motif of the JAK2 kinase, nucleotide sequences encoding the corresponding domains of JAK2 are removed from the JAK2 coding sequences by standard recombinant DNA techniques. The truncated DNA is inserted into a recombinant expression vector, which is then introduced

into a cell to allow for expression of the truncated JAK2, lacking the YXXL motif, in the cell.

Other inhibitory agents that can be used to inhibit the expression and/or activity of a JAK2 kinase include chemical compounds that directly inhibit a JAK2 kinase or compounds that inhibit the interaction between a JAK2 and target DNA or another polypeptide. Such compounds can be identified using screening assays that select for such compounds, as described herein.

II. Pharmaceutical Compositions

As described herein, the JAK2 inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation described herein can be used primarily in therapeutic methods for the treatment or prevention of RNA polymerase dependent viral disorders, such as HIV, influenza, hepatitis and west nile virus. As described herein, JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation are involved in the modulation of the phosphorylation of HEXIM1, modulation of the release of HEXIM1 for the pTEFb complex, modulation of pTEFb complex activity, modulation of TAT interaction with RNA polymerase, modulation of CDK9 mediated phosphorylation of RNA polymerase, modulation of RNA polymerase activity, modulation of the transcription of the viral genome, modulation of the replication of the viral genome, and modulation of the spread of a RNA polymerase dependent virus.

The JAK2 inhibitory agents or agents that inhibit HEXIM1 tyrosine phosphorylation of the present invention, for example, nucleic acid molecules, polypeptides, antibodies, or portions thereof, or other inhibitory agents of the invention, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, or polypeptide and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal

(topical), transmucosal, vaginal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the JAK2 kinase inhibitor (*e.g.*, AG490, small molecules such as Z3, antisense molecules and anti-JAK2 antibody) or agents that inhibit HEXIM1 tyrosine phosphorylation in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of

preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery. Vaginal suppositories or foams for local mucosal delivery may also be prepared to block sexual transmission.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be

obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens and liposomes targeted to macrophages containing, for example, phosphatidylserine) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811 and U.S. Patent No. 5,643,599, the entire contents of which are incorporated herein.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease, disorder, or infection, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can include a single treatment or, preferably, can include a series of treatments.

In a preferred example, a subject is treated with antibody or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of the JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

As described herein, the present invention encompasses JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation which modulate expression or activity. A JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*,. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Exemplary doses include milligram or microgram amounts of the JAK2 inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation, *e.g.*, small molecule, per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologues thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A,

pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moieties to antibodies are well known, see, *e.g.*, Arnon, *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld, *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom, *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson, *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin, *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe, *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982). Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen, *et al.* (1994) *Proc. Natl. Acad. Sci., USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

III. Methods of the Invention

Therapeutic Methods

The present invention is generally directed to a method for treating or preventing a viral disorder in a subject having or at risk of having said viral disorder, wherein said virus is RNA polymerase dependent, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation, thereby treating or preventing the viral disorder (for example, HIV, influenzavirus, influenzavirus A such as H1N1, influenzavirus B, influenzavirus C, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus) in said subject. In another aspect, the present invention is directed to a method for inhibiting the replication of a virus in a subject, by administering to the subject infected with said virus, wherein said virus is dependent on RNA polymerase for replication (for example, HIV, influenzavirus, influenzavirus A such as H1N1, influenzavirus B, influenzavirus C, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus), an effective amount of a composition including a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation, thereby inhibiting viral replication in the subject.

The methods of the present invention are based, at least in part, on the elucidation of the role of JAK2 kinase and the tyrosine phosphorylation of HEXIM1 in the replication of RNA polymerase dependent viruses. Specifically, the inventors have identified that JAK2 kinase and the tyrosine phosphorylation of HEXIM1 at its YLEL epitope play an important role in activating RNA polymerase II so as to promote viral gene expression and viral replication after infection of a cell. Accordingly, by inhibiting JAK2 kinase or the tyrosine phosphorylation of HEXIM1 at the YLEL epitope, the inventors have discovered that viral replication can be inhibited, thereby providing a method of treating RNA polymerase dependent virus based disorders such as HIV, Influenza, Hepatitis C and West Nile Virus.

In particular, the inventors have identified that that JAK2 kinase serves to phosphorylate HEXIM1, specifically, at the YLEL epitope present on HEXIM1, so as to trigger the release of HEXIM1 from the transcription elongation factor P-TEFb complex (cyclin T1-cdk9). Upon release of HEXIM1 from the complex, a viral replicating factor, for example, TAT (an HIV replicating factor), complexes with the P-TEFb complex thereby hijacking the host cell's transcriptional machinery for purposes of promoting viral replication. Indeed, upon the formation of the viral replicating factor with P-TEFb complex, the P-TEFb complex, specifically, cdk9, triggers phosphorylation and transcription elongation of RNA Polymerase II so as to allow for transcription and replication of the viral genome.

Accordingly, based on this elucidated mechanism of action and, in particular, the JAK2 kinase phosphorylation of HEXIM1 at the YLEL motif, the inventors have identified that RNA polymerase dependent virus based disorders, including lentivirus based disorders, such as Human Immunodeficiency Virus (HIV) (HIV-1 and HIV-2), Influenza (Influenzavirus A such as H1N1, Influenzavirus B and Influenzavirus C), Hepatitis (Hepatitis virus A, Hepatitis virus C, Hepatitis virus D and Hepatitis virus E), and the West Nile Virus, can be treated by use of JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation at the YLEL motif.

Accordingly, the subject methods employ agents that inhibit JAK2 expression, processing, post-translational modification, or activity, or the expression, processing, post-translational modification, or activity of another molecule in a JAK2 signaling pathway, *e.g.,* HEXIM1, such that JAK2 or the activity of a molecule in a JAK2 signal transduction pathway is modulated, for example, inhibited. The subject methods are useful in both clinical and non-clinical settings.

In one embodiment, the instant methods can be performed *in vitro.* In another embodiment, the instant methods can be performed in a cell *in vitro* and then the treated cell can be administered to a subject.

The term "subject" is intended to include living organisms in which an immune response can be elicited. Preferred subjects are mammals. Particularly preferred subjects are humans. Other examples of subjects include monkeys, dogs, cats, mice, rats, cows, horses, goats, sheep as well as other farm and companion animals. Inhibition of JAK2 expression and/or activity or inhibition of HEXIM1 tyrosine phosphorylation, in humans as well as veterinary applications, provides a means to regulate RNA polymerase dependent viral disorders arising from JAK2 expression and/or activity or HEXIM1 tyrosine phosphorylation in various disease states and is encompassed by the present invention.

Identification of compounds that inhibit the biological effects of JAK2 or the tyrosine phosphorylation of HEXIM1 by directly or indirectly inhibiting JAK2 expression and/or activity or HEXIM1 tyrosine phosphorylation allows for selective manipulation of these biological effects in a variety of clinical situations using the methods of the invention. For example, the inhibitory methods of the invention can operate by modulating the phosphorylation of JAK2 protein, the phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex, the activity of pTEFb complex, TAT interaction with RNA polymerase, CDK9 mediated phosphorylation of RNA polymerase, the activity of RNA polymerase activity and/or the transcription of the viral genome.

Application of the inhibitory methods of the invention to the treatment of a disorder may result in cure of the disorder, a decrease in the type or number of symptoms associated

with the disorder, either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Treatment and/ or Prevention of HIV

In a particular aspect, the present invention is directed to a method for treating or preventing HIV infection in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1 tyrosine phosphorylation, thereby treating or preventing HIV in said subject. In yet another aspect, the present invention includes methods for inhibiting the replication of HIV in a subject having or at risk of HIV infection, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1 tyrosine phosphorylation, thereby inhibiting HIV replication in the subject. The HIV may either be HIV-1 or HIV-2.

The present invention is based, at least in part, on the identification of the role that JAK2 kinase plays in activating RNA polymerase, thereby allowing for the expression, replication and spread of the HIV genome. Specifically, RNA polymerase II serves to catalyze the replication of HIV genomic material and, further, to generate viral mRNA utilized to generate HIV proteins during translation. Upon HIV infection of a cell, the single stranded HIV RNA is converted to double-stranded HIV DNA which subsequently enters the host cell's nucleus and integrates within the host cell's DNA. The integrated viral DNA is referred to as a provirus. Upon activation of the host cell, the host cell's endogenous RNA polymerase is hijacked by the provirus so as to catalyze the replication of HIV genomic material and, further, to generate viral mRNA. Specifically, TAT, an HIV encoded transcription factor, binds to the TAT-Responsive element (TAR) in the viral RNA, prompting CDK9 to phosphorylate RNA polymerase II, which in turn serves to continue replication and elongation of the viral RNA.

As set forth above, JAK2 kinase serves to activate the RNA polymerase to catalyze the replication of HIV genomic material and, further, to generate viral mRNA utilized to generate HIV proteins during translation. Specifically, JAK2 kinase serves to phosphorylate HEXIM1 at the YLEL epitope present on HEXIM1, so as to trigger the release of HEXIM1 from the transcription elongation factor P-TEFb complex (cyclin T1-cdk9). The c-termini of HEXIM1 (KQELIKEYLELEKCLS) plays a key role in both the phosphorylation of HEXIM1 by JAK2 kinase and the interaction of HEXIM1 with cyclin T1. First, the post-translational modification of HEXIM1, *i.e.*, the tyrosine phosphorylation of the YLEL motif (amino acid residues 296-299) of HEXIM1 by JAK2 kinase, facilitates the release of HEXIM1 from cyclin T1 at the KEYL motif (amino acid residues 289-292)

of HEXIM1 (see Dames *et al.* PNAS (2007) 104(36): 14312-14317), the contents of which are hereby incorporated herein by reference).

The release of HEXIM1 from the p-TEFb complex and, in particular, from the cyclin T1, enables TAT (an HIV replicating factor) to complex with the P-TEFb complex thereby hijacking the host cell's transcriptional machinery for purposes of promoting viral replication. Indeed, upon the formation of the TAT - P-TEFb complex, the P-TEFb complex, specifically, cdk9, triggers phosphorylation and transcription elongation of RNA Polymerase II so as to allow for transcription and replication of the HIV genome.

Accordingly, inhibition of JAK2 kinase activity or inhibition of the tyrosine phosphorylation of HEXIM1 should serve to lock the access of tat to cyclinT1, thereby inhibiting HIV replication.

Based, in part, on the foregoing mechanism, the present invention sets forth that the inhibition of JAK2 through administration of a JAK2 inhibitor or, alternatively, the inhibition of HEXIM1 tyrosine phosphorylation through administration of an agent effecting such activity, can serve to treat and/or prevent HIV. For example, in eliciting the desired therapeutic or prophylactic effect, the JAK2 kinase inhibitor can operate by modulating the tyrosine phosphorylation of JAK2 protein, the tyrosine phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex, the activity of pTEFb complex, TAT interaction with RNA polymerase, CDK9 mediated serine phosphorylation of RNA polymerase, the activity of RNA polymerase activity and/or the transcription of the viral genome.

Treatment and/or Prevention of Influenza

In another aspect, the present invention is directed to a method for treating or preventing influenza in a subject having or at risk of infection by an influenzavirus, for example, any of influenza A (*e.g.*, H1N1) , B or C, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1 tyrosine phosphorylation, thereby treating or preventing influenza in said subject. In yet another aspect, the present invention includes methods for inhibiting the replication of the influenza virus, for example, influenza A (*e.g.*, H1N1), B or C, in a subject having or at risk of infection by an influenzavirus, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1 tyrosine phosphorylation, thereby inhibiting replication of the influenza virus in the subject.

The present invention is based, at least in part, on the identification of the role of JAK2 kinase in the replication of influenzaviruses. (-) strand influenza viral RNAs enter the nucleus, where they serve as templates for the synthesis of mRNA's for the synthesis of viral proteins in the cytoplasm. The RNA-dependent RNA polymerase carried by the virus, is critical for translating the (-) strand viral RNAs into protein, particularly in light of the fact that the host cells has no enzymes which can copy such long RNA molecules.

Additionally, RNA polymerases, specifically, RNA dependent RNA polymerase, trigger the production of additional (-) strand RNAs necessary for assembling new virions. Specifically, RNA polymerases, carried by the influenzavirus, helps in producing a full length (+) strand, which, in turn, is copied to a full-length (-) strand RNA for use in assembling new virions.

Transcription by the influenza virus RNA-dependent RNA polymerase is dependent on cellular RNA processing activities that are known to be associated with cellular RNA polymerase II (Pol II) transcription, namely, capping and splicing. The influenza virus RNA polymerase complex interacts with the large subunit of Pol II via its C-terminal domain. The viral polymerase binds hyperphosphorylated forms of Pol II, indicating that it targets actively transcribing Pol II. In addition, immunofluorescence analysis is consistent with a new model showing that influenza virus polymerase accumulates at Pol II transcription sites. Accordingly, regulation of RNA polymerase II activities, for example, by administration of JAK2 kinase inhibitors or agents that inhibit the tyrosine phosphorylation of HEXIM1, can serve to modulate influenza virus replication, thereby treating influenza virus infection.

As set forth above, JAK2 kinase serves to activate the RNA polymerase to catalyze the replication of the influenza genomic material and, further, to generate viral mRNA utilized to generate HIV proteins during translation. Specifically, JAK2 kinase serves to phosphorylate HEXIM1, specifically, at the YLEL epitope present on HEXIM1, so as to trigger the release of HEXIM1 from the transcription elongation factor P-TEFb complex (cyclin T1-cdk9). Upon release of HEXIM1 from the complex, the P-TEFb complex hijacks the host cell's transcriptional machinery for purposes of promoting replication of the influenzavirus.

Accordingly, inhibiting JAK2 through administration of a JAK2 inhibitor or, alternatively, inhibiting HEXIM1 tyrosine phosphorylation through administration of an agent effecting such activity, can serve to treat and/or prevent influenza. For example, in eliciting the desired therapeutic or prophylactic effect, the JAK2 kinase inhibitor or agent that inhibits tyrosine phosphorylation of HEXIM 1 can operate by modulating the phosphorylation of JAK2 protein, the phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex, the activity of pTEFb complex, CDK9 mediated phosphorylation

of RNA polymerase, the activity of RNA polymerase activity and/or the transcription of the influenza virus genome.

Treatment and/or Prevention of Hepatitis

In another aspect, the present invention is directed to a method for treating or preventing hepatitis in a subject having or at risk of infection by an influenza virus, for example, any of influenza A (*e.g.*, H1N1), C, D or E, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1tyrosine phosphorylation, thereby treating or preventing hepatitis in said subject. In yet another aspect, the present invention includes methods for inhibiting the replication of the hepatitis virus, for example, hepatitis A, C, D or E, in a subject having or at risk of infection by hepatitis, by administering to the subject an effective amount of a composition including a JAK2 kinase inhibitor or an agent that inhibits HEXIM1 tyrosine phosphorylation, thereby inhibiting replication of the hepatitis virus in the subject.

According to the present invention, JAK2 kinase plays an important role in the replication of various hepatitis viruses. As set forth above, JAK2 kinase plays an important role in activating RNA polymerase to effect viral expression and replication, a role that is equally important with respect specifically to hepatitis viruses.

For example, Hepatitis A and E utilize a virus encoded RNA-dependent RNA polymerase to replicate their respective genomes. Hepatitis C additionally utilizes an RNA dependent RNA polymerase, for example, NS5B, to effect viral replication. Hepatitis D utilizes RNA polymerase II to copy its genome, specifically replicating (-) strand viral RNA to a (+) strand RNA.

Accordingly, inhibiting JAK2 through administration of a JAK2 inhibitor or, alternatively, inhibiting HEXIM1 tyrosine phosphorylation through administration of an agent effecting such activity, can serve to treat and/or prevent hepatitis by inhibiting RNA polymerase activity. For example, in eliciting the desired therapeutic or prophylactic effect, the JAK2 kinase inhibitor or agent inhibiting HEXIM1 tyrosine phosphorylation can operate by modulating the phosphorylation of JAK2 protein, the phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex, the activity of pTEFb complex, CDK9 mediated phosphorylation of RNA polymerase, the activity of RNA polymerase activity and/or the transcription of the hepatitis virus genome.

Administration of JAK2 kinase inhibitors or agents that inhibits HEXIM1 tyrosine phosphorylation

JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to effect the desired therapeutic effect, for example, to treat HIV infection by inhibiting viral replication. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the modulating agent. The term subject is intended to include living organisms in which an immune response can be elicited, *e.g.,* mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including for example intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in ex vivo treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation.

The a JAK2 kinase inhibitor can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can be coupled to any substance known in the art to promote infection of HIV infected cells such as an antibody specific for receptors on such infected cells, and administered by intravenous injection. Furthermore, a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See for example Davis *et al.*, 1978, *Enzyme Eng* 4: 169-73; Burnham, 1994, *Am J Hosp Pharm* 51: 210-218, which are incorporated by reference).

Furthermore, the a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can be in a composition which aids in delivery into the cytosol of a cell. For example, the agent may be conjugated with a carrier moiety such as a liposome that is capable of delivering the peptide into the cytosol of a cell. Such methods are well known in the art (for example see Amselem *et al.*, 1993, *Chem Phys Lipids* 64: 219-237, which is incorporated by reference). Alternatively, the a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can be modified to include specific transit peptides or fused to such transit peptides which are capable of delivering the JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation into a cell. In addition, the agent can be delivered directly into a cell by microinjection.

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation can also be incorporated into a solid or semi-solid biologically compatible matrix which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used. It is also

provided that certain formulations containing the JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, olyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals. The specific dose can be readily calculated by one of ordinary skill in the art, *e.g.,* according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in assay preparations of target cells. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

For inhibitory agents that comprise nucleic acids (including recombinant expression vectors encoding JAK2 inhibitors, antisense RNA, or dominant negative inhibitors), the agents can be introduced into cells of the subject using methods known in the art for introducing nucleic acid (*e.g.*, DNA) into cells *in vivo*. Examples of such methods encompass both non-viral and viral methods, including:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly injecting the DNA into the cells (see *e.g.*, Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (*e.g.*, a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (*e.g.*, from BioRad).

Cationic Lipids: Naked DNA can be introduced into cells *in vivo* by complexing the DNA with cationic lipids or encapsulating the DNA in cationic liposomes. Examples of suitable cationic lipid formulations include N-[-1-(2,3-dioleoyloxy)propyl]N,N,N-triethylammonium chloride (DOTMA) and a 1:1 molar ratio of 1,2-dimyristyloxy-propyl-3-dimethylhydroxyethylammonium bromide (DMRIE) and dioleoyl phosphatidylethanolamine (DOPE) (see *e.g.*, Logan, J.J. *et al.* (1995) *Gene Therapy* 2:38-49; San, H. *et al.* (1993) *Human Gene Therapy* 4:781-788).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of

suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991) *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 *etc.*) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors

currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al. Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (*e.g.*, Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product.

In a preferred embodiment, a retroviral expression vector encoding a JAK2 kinase inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation is used to express the inhibitor in cells *in vivo*. Such retroviral vectors can be prepared according to standard methods known in the art (discussed further above).

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such

compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method for the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Monitoring the influence of JAK2 kinase inhibitors or agents that inhibit HEXIM1 phosphorylation (*e.g.,* drugs or compounds) on the expression or activity of a JAK2 kinase or HEXIM1 can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to decrease JAK2 gene expression, protein levels, or to downregulate JAK2 activity, can be monitored in clinical trials of subjects having RNA polymerase viral disorders. In such clinical trials, the expression or activity of a JAK2 gene, and preferably, other genes that have been implicated in a disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including JAK2 encoding genes, that are modulated in cells by treatment with a JAK2 kinase inhibitor (*e.g.,* compound, drug or small molecule) which modulates JAK2 activity (*e.g.,* identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on a JAK2 associated disorder including viral disorders such as HIV, influenza, west nile virus and hepatitis, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of JAK2 and other genes implicated in the viral JAK2 associated disorder, respectively. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of JAK2 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.,* an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a JAK2 protein, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the JAK2 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the JAK2 protein, mRNA, or genomic DNA in the pre-administration sample with the JAK2 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly.

In a preferred embodiment, the ability of a JAK2 inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation to modulate RNA polymerase dependent viral infection, for example, HIV, influenza, hepatitis and west nile virus, in a subject can be measured by detecting an improvement in the condition of the patient after the administration of the inhibitor. Such improvement can be readily measured by one of ordinary skill in the art using indicators appropriate for the specific condition of the patient. Monitoring the response of the patient by measuring changes in the condition of the patient is preferred in situations where the collection of biopsy materials would pose an increased risk and/or detriment to the patient.

In one embodiment, tyrosine-phospho-HEXIM1 antibodies could be used in an ELISA test to monitor viral replication by measuring the level of tyrosine phosphorylated YXXL-Hexim1 in subjects.

Furthermore, in the treatment of disease conditions, compositions containing a JAK2 inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation can be administered exogenously and it would likely be desirable to achieve certain target levels of JAK2 inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation in sera, in any desired tissue compartment or in the affected tissue. It would, therefore, be advantageous to be able to monitor the levels of the JAK2 inhibitor or agent that inhibits HEXIM1 tyrosine phosphorylation in a patient or in a biological sample including a tissue biopsy sample obtained form a patient and, in some cases, also monitoring the levels of JAK2, and/or, HEXIM1, pTEFb complex or other upstream or downstream JAK2 interacting proteins. Accordingly, the present invention also provides methods for detecting the presence of JAK2 in a sample from a patient.

Screening Assays

The invention further provides methods (also referred to herein as "screening assays") for identifying further JAK2 kinase inhibitors or agents for preventing the tyrosine phosphorylation of HEXIM1, *i.e.*, candidate or test compounds or agents (*e.g.,* peptidomimetics, small molecules or other drugs) which modulate, for example one or more JAK2 activity or upstream or downstream activities thereof, *e.g.,* the tyrosine phosphorylation of JAK2 protein, the tyrosine phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex (for example, at the YXXL motif), the modulation of pTEFb complex activity, the modulation of TAT interaction with RNA polymerase, the modulation of CDK9 mediated phosphorylation of RNA polymerase, the modulation of RNA polymerase activity, the modulation of the transcription of the viral genome, the modulation of the replication of the viral genome, and the modulation of the spread of a virus.

The assays can be used to identify agents that modulate, in particular, inhibit, the function of JAK2 and/or a JAK2-binding molecule, such as, but not limited to HEXIM1. For example, such agents may interact with JAK2 or the JAK2-binding molecule (*e.g.,* to inhibit their activity). The function of JAK2 or the JAK2-binding molecule can be affected at any level, including transcription, protein expression, protein localization, and/or cellular activity. The subject assays can also be used to identify, *e.g.,* agents that alter the interaction of JAK2 or the JAK2-binding molecule with a binding partner, substrate, or cofactors, or modulate, *e.g.,* decrease, the stability of such interaction.

The subject screening assays can measure the activity of JAK2 or a JAK2-binding protein directly (*e.g.,* phosphorylation or ubiquitination), or can measure a downstream event controlled by modulation of JAK2 or a JAK2-binding protein (*e.g.,* the phosphorylation of JAK2 protein, the phosphorylation of HEXIM1, the release of HEXIM1 for the pTEFb complex (for example, at the YXXL motif), the modulation of pTEFb complex activity, the modulation of TAT interaction with RNA polymerase, the modulation of CDK9 mediated phosphorylation of RNA polymerase, the modulation of RNA polymerase activity, the modulation of the transcription of the viral genome, the modulation of the replication of the viral genome, and the modulation of the spread of a virus).

The subject screening assays employ indicator compositions. These indicator compositions comprise the components required for performing an assay that detects and/or measures a particular event. The indicator compositions of the invention provide a reference readout and changes in the readout can be monitored in the presence of one or more test compounds. A difference in the readout in the presence and the absence of the compound indicates that the test compound is a modulator of the molecule(s) present in the indicator composition.

The indicator composition used in the screening assay can be a cell that expresses a JAK2 or a JAK2-binding molecule. For example, a cell that naturally expresses or, more preferably, a cell that has been engineered to express the protein by introducing into the cell an expression vector encoding the protein may be used. Alternatively, a cell that is preferentially infected by the virus in question can be used. Preferably, the cell is a mammalian cell, *e.g.,* a human cell. With respect to the identification of JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation for the treatment of HIV infection, the cell can be either a T lymphocyte or macrophage. Alternatively, the indicator composition can be a cell-free composition that includes the protein (*e.g.,* a cell extract or a composition that includes *e.g.,* either purified natural or recombinant protein).

The indicator composition used in the screening assays of the invention can be a cell that expresses a JAK2 kinase and/or HEXIM1, for example, as set forth in Figure 1, or biologically active fragment thereof.

In another embodiment, the indicator composition comprises more than one polypeptide. For example, in one embodiment the subject assays are performed in the presence of JAK2 kinase and/or at least one JAK2-binding molecule, such as, but not limited to HEXIM1.

Compounds that modulate, for example, inhibit, the expression and/or activity of JAK2, identified using the assays described herein can be useful for treating a subject suffering from RNA polymerase dependent viral disorders that would benefit from the inhibition of JAK2 production.

In one embodiment, secondary assays can be used to confirm that the inhibiting agent affects the JAK2 molecule or the HEXIM1 in a specific manner. For example, compounds identified in a primary screening assay can be used in a secondary screening assay to determine whether the compound inhibits a JAK2-related activity or HEXIM1 tyrosine phosphorylation. Accordingly, in another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, an inhibitor can be identified using a cell-based or a cell-free assay, *e.g.,* to detect binding, and the ability of the agent to modulate the activity of JAK2 can be confirmed using a biological read-out to measure, *e.g.*, HEXIM1 phosphorylation, *in vitro* or *in vivo*.

Moreover, JAK2 kinase inhibitors or agents that inhibit HEXIM1 tyrosine phosphorylation identified as described herein (*e.g.,* a small molecule) may be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an inhibitor. Alternatively, an inhibitor identified as described herein may be used in an animal model to determine the mechanism of action of such inhibitor.

In one embodiment, the screening assays of the invention are high throughput or ultra high throughput (*e.g.,* Fernandes, P.B., *Curr Opin Chem Biol.* 1998 2:597; Sundberg, SA, *Curr Opin Biotechnol.* 2000, 11:47).

Test Compounds

A variety of test compounds can be evaluated using the screening assays described herein. The term "test compound" includes any reagent or test agent which is employed in the assays of the invention and assayed for its ability to influence either the production, expression and/or activity of JAK2 kinase or the tyrosine phosphorylation of HEXIM1. More than one compound, *e.g.,* a plurality of compounds, can be tested at the same time for their ability to modulate cytokine production, expression and/or activity in a screening assay. The term "screening assay" preferably refers to assays which test the ability of a plurality of compounds to influence the readout of choice rather than to tests which test the ability of one compound to influence a readout. Preferably, the subject assays identify compounds not previously known to have the effect that is being screened for. In one embodiment, high throughput screening may be used to assay for the activity of a compound.

In certain embodiments, the compounds to be tested can be derived from libraries (*i.e.*, are members of a library of compounds). While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures of other compounds, such as benzodiazepines (Bunin, *et al.* (1992). *J. Am. Chem. Soc*. 114:10987; DeWitt *et al.* (1993). *Proc. Natl. Acad. Sci., USA* 90:6909) peptoids (Zuckermann. (1994). *J. Med. Chem.* 37:2678) oligocarbamates (Cho, *et al.* (1993). *Science*. 261:1303), and hydantoins (DeWitt, *et al. supra*). An approach for the synthesis of molecular libraries of small organic molecules with a diversity of 104-105 has been described (Carell, *et al.* (1994). *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell, *et al.* (1994) Angew. *Chem. Int. Ed. Engl*. 33:2061).

The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145). Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: Erb, *et al.* (1994). *Proc. Natl. Acad. Sci.,*

USA 91:11422- ; Horwell, *et al.* (1996) *Immunopharmacology* 33:68-; and in Gallop, *et al.* (1994); *J. Med. Chem.* 37:1233.

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

Candidate/test compounds include, for example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, *e.g.,* Lam, K.S., *et al.* (1991) *Nature* 354:82-84; Houghten, R., *et al.* (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids; 2) phosphopeptides (*e.g.,* members of random and partially degenerate, directed phosphopeptide libraries, see, *e.g.,* Songyang, Z., *et al.* (1993) *Cell* 72:767-778); 3) antibodies (*e.g.,* antibodies (*e.g.,* intracellular, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments of antibodies); 4) small organic and inorganic molecules (*e.g.,* molecules obtained from combinatorial and natural product libraries); 5) enzymes (*e.g.,* endoribonucleases, hydrolases, nucleases, proteases, synthatases, isomerases, polymerases, kinases, phosphatases, oxido-reductases and ATPases), and 6) mutant forms of molecules (*e.g.,* dominant negative mutant forms of JAK2 or a JAK2-binding protein).

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt, *et al.* (1993) *Proc. Natl. Acad. Sci., U.S.A.* 90:6909; Erb, *et al.* (1994) *Proc. Natl. Acad. Sci., USA* 91:11422; Zuckermann, *et al.* (1994) *J. Med. Chem.* 37:2678; Cho, *et al.* (1993) *Science* 261:1303; Carrell, *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell, *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop, *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.,* Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner US Patent No. 5,223,409), spores (Ladner

US Patent No. '409), plasmids (Cull, *et al.* (1992) *Proc. Natl. Acad. Sci., USA* 89:1865-1869) or phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla, *et al.* (1990) *Proc. Natl. Acad. Sci., USA* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner *supra.*).

Compounds identified in the subject screening assays may be used, *e.g.,* in methods of modulating phosphorylation of JAK2 protein, phosphorylation of HEXIM1, release of HEXIM1 for the pTEFb complex, pTEFb complex activity, TAT interaction with RNA polymerase, CDK9 mediated phosphorylation of RNA polymerase, RNA polymerase activity, modulation of the transcription of the viral genome, the replication of the viral genome, and the spread of a virus. It will be understood that it may be desirable to formulate such compound(s) as pharmaceutical compositions (described supra) prior to contacting them with cells.

Once a test compound is identified that directly or indirectly modulates, *e.g.,* production, expression and/or activity of a gene regulated by JAK2 and/or a JAK2-binding molecule, for example, HEXIM1, by one of the variety of methods described herein, the selected test compound (or "compound of interest") can then be further evaluated for its effect on cells, for example by contacting the compound of interest with cells either *in vivo* (*e.g.,* by administering the compound of interest to a subject) or *ex vivo* (*e.g.,* by isolating cells from the subject and contacting the isolated cells with the compound of interest or, alternatively, by contacting the compound of interest with a cell line) and determining the effect of the compound of interest on the cells, as compared to an appropriate control (such as untreated cells or cells treated with a control compound, or carrier, that does not modulate the biological response).

The instant invention also pertains to compounds identified in the subject screening assays.

IV. Kits of the Invention

Another aspect of the invention pertains to kits for carrying out the screening assays, modulatory methods or diagnostic assays of the invention. For example, a kit for carrying out a screening assay of the invention can include an indicator composition comprising a JAK2 kinase and/or HEXIM1, means for measuring a readout (*e.g.*, protein secretion) and instructions for using the kit to identify modulators of biological effects of JAK2 or HEXIM1 tyrosine phoshorylation. In another embodiment, a kit for carrying out a screening assay of the invention can include cells deficient in JAK2 or HEXIM1, means for measuring the readout and instructions for using the kit to identify modulators of a biological effect of JAK2 or HEXIM1 tyrosine phosphorylation.

For example, the kit may further include a tyrosine phospho-HEXIM1 antibody for use in an ELISA assay to measure the level of HEXIM1 phosphorylated at the tyrosine residue of the YLEL epitope. In another embodiment, the invention provides a kit for carrying out a modulatory method of the invention. The kit can include, for example, a modulatory agent of the invention (*e.g.*, a JAK2 kinase inhibitor or an agent that inhibits HEXIM1) in a suitable carrier and packaged in a suitable container with instructions for use of the inhibitor to modulate a biological effect of JAK2 or HEXIM1 tyrosine phosphorylation or to treat an RNA polymerase dependent viral disorder such as HIV, Influenza, Hepatitis or West Nile Virus.

Another aspect of the invention pertains to a kit for diagnosing a disorder associated with a biological activity of a JAK2 kinase or HEXIM1 in a subject. The kit can include a reagent for determining expression of JAK2 (*e.g.*, a nucleic acid probe for detecting JAK2 mRNA or an antibody for detection of JAK2 protein) or tyrosine phosphorylation of HEXIM1 (*e.g.*, a tyrosine phospho-HEXIM1 antibody), a control to which the results of the subject are compared, and instructions for using the kit for diagnostic purposes.

This invention is further illustrated by the following example, which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

EXAMPLES

Example 1: Inhibition of HIV replication by JAK2 kinase inhibitor AG490 in cultured human Jurkat cells

Materials:

The following materials were utilized in assaying the inhibition of HIV replication by the JAK2 kinase inhibitor AG490.

1. Jurkat cells, (ATCC No. 10915™)
2. DMEM cell culture medium, supplemented with 10% Fetal Bovine Serum, heat inactivated, 1mM Pyruvate- Jurkat cell (Invitrogen).
3. HIV-1 delta nef virus: 200-bp nef deleted HIV-1 Eli virus – frozen at -80°C (IDI, Inc.)

4. AG490 (Santa Cruz, Sigma or LC Labratories).
5. Tissue culture plates, plastics, processing equipment, centrifuges.
6. HIV-1 p24 Elisa assay kit (IDI, Inc. product # 103)
7. HIV-1 p24 one-step cassette test kit (IDI, Inc. product # IT200)

Inhibitor Assay Method:

Confluent cultures of Jurkat cells at 2-3X10^6 cells per ml, 98% viable were acquired. Cells were diluted to 5X10^5 cells per ml with media as defined in Materials 2 above. 1 ml diluted Jurkat cells were transferred to 4 wells x 2 rows each of a 24 tissue culture plate. The plate was subsequently transferred to a precision 37°C incubator, 10% CO_2 for 1 hour.

A vial of HIV-1 delta nef Eli frozen virus was removed from the -75°C freezer.

A 100 micro-molar solution of AG490 inhibitor was prepared as follows. Initially a 1 mg/ml solution of AG490 in DMSO was prepared. Using a syringe and 22G needle, 0.2ml of the DMSO stock was removed and diluted with GMEM medium to 2 ml. The solution was filter sterilized by passage through a 0.2 micron disc filter (Millipore).

The tissue culture plate from the incubator was placed under the sterile hood and surface swiped with 70% ethanol. Each well of the duplicate cultures was labeled as follows: (1) Blank, B; (2) delta nef virus, DNV; (3) AG490, AG; and (4) Delta nef virus AG490, VAG.

100ul of sterile AG490 was added to the AG and VAG wells. 100µl of delta nef virus was added to the DNV and VAG wells. Appropriate media was subsequently added to the wells in amounts to bring up the volume to 2ml per well.

Lastly, the culture plate was returned to the 37°C incubator, 10% CO_2.

On day 8, all the wells were completely exchanged with appropriate fresh media and AG490 was replenished in wells AG and VAG at 5 micro-molar concentration.

p24 antigen was assayed in culture supernatants on days 8, 10, 13, 15, 18 and 21 using a p24 Elisa kit (Product # 103 from IDI, Inc.) following the kit instructions as follows. The culture plate was transferred to the safety hood and swiped carefully with 70% EtOH. With a micropippetor and sterile tip, 100µl of culture supernatant was removed from each well set of Jurkat wells and transferred to an Eppendorf tube containing 20µl of a 10X solution of PBST (PBS+ 0.05% Tween 20). The Eppendorf tubes were capped and transferred to a 55°C water bath for 10 minutes. Subsequently, the Eppendorf tubes were returned to the sterile hood and diluted with heat inactivated culture supernatants to a final volume of 220µl with PBSTB

(PBS+ Tween+1% BSA). Finally 100 µl of each sample was transferred in duplicate to p24 Elisa plate wells where the manufacturer kit instructions were followed for the p24 antigen assay.

The p24 antigen assay was conducted on days 8, 10, 13, 15, 18 and 21 as described above in order to determine p24 antigen levels in the culture supernatants. Results were recorded using BioRad Gel Doc system.

Results:

The results of the p24 assay were as follows:

Days Post Infection	P24 antigen Elisa Result (OD 450 nm) Jurkat Cells		
	-AG490	+AG490	% Inhibition
Day 8	0.2	0.08	-
Day 10	0.37	0.11	30
Day 13	0.83	0.12	86
Day 17	1.13	0.12	90
Day 21	2.0	0.15	92

Conclusions:

As depicted above, the results reflect a clear inhibition of HIV replication upon administration of JAK2 kinase inhibitor AG490. Specifically, the results depict 92% inhibition of HIV-1 replication in Jurkat cells, thereby demonstrating that AG490 can be used to inhibit HIV-1 replication. Finally, we note that the AG490 half-life may not be more than 48 hours.

Example 2: Inhibition of Endogenous HIV replication by JAK2 kinase inhibitor Z3 in cultured human Hut-78 (H9) cells

Experimental Summary:

Confluent H9 cells were diluted in DMEM + 10% fetal calf serum (FCS) to a cell density of approximately $1x10^6$/ml, and 1ml cells were seeded per well in 6-well culture

dishes. DMSO-vehicle, Z3 in DMSO and HIV-1 virus were added to wells as follows. DMSO-vehicle (C)), Z3 in DMSO, (Z3), DMSO-vehicle+ HIV-1 virus (D-nef), and Z3 in DMSO + D-nef virus (D-nef-Z3). The culture dishes were incubated at 37°C, 10% CO_2, for 8 days. On day 8, culture supernatant p24 antigen was assayed by p24 Lateral Flow Antigen test. Residual p24 was detected in HIV-1 infected cells, without and with Z3.

On day 8, cells were given a complete medium exchange, DMSO-vehicle was replenished in (C) and D-nef wells, and Z3 inhibitor added to Z3 and D-nef + Z3 wells. D-nef and D-nef+ Z3 cells were not re-infected with D-nef HIV-1 on day 8. The cultures were visually monitored on days 10, 13, 17, and endogenous virus replication was determined by measuring p24 antigen in culture supernatants on day 22 as before.

Materials:

The following materials were utilized in assaying the inhibition of HIV replication by the JAK2 kinase inhibitor Z3.

1. Hut-78 (H9) cells (ATCC No. HTB-176™)
2. DMEM cell culture medium, supplemented with 10% Fetal Bovine Serum, heat inactivated – H9 cell (Invitrogen).
3. HIV-1 delta nef virus: 200-bp nef deleted HIV-1 Eli virus – frozen at -80°C (IDI, Inc.).
4. Z3 Jak2 kinase inhibitor (Santa Cruz Labs.)
5. Tissue culture plates, plastics, processing equipment, centrifuges.
6. HIV-1 p24 one-step cassette test kit (IDI, Inc. product # IT200).

Inhibitor Assay Method:

Confluent cultures of H9 cells at 2-3X106 cells per ml, 98% viable were acquired. Cells were diluted to $1X10^6$ cells per ml. H9 cells were appropriately diluted with media as defined above. 1 ml diluted H9 cells were transferred to 4 wells each of a 24 tissue culture plate. The plate was subsequently transferred to a precision 37°C incubator, 10% CO_2 for 1 hour.

A vial of HIV-1 delta nef Eli frozen virus is removed from the -75°C freezer.

A 10 micro-molar solution of Z3 inhibitor was prepared as follows. Initially a 1 mg/ml solution Z3 in DMSO was prepared. The Z3 solution was filter-sterilized using a 0.2 micron disc filter (Millipore). The 10 micro-molar solution is stored at 4°C.

The H9 tissue culture plate from the incubator was placed under the sterile hood. The plate was surface swiped with 70% ethanol. Each well of the duplicate cultures was labeled as follow: (1) Blank, B; (2) delta nef virus, DNV; (3) Z3; and (4) Delta nef virus Z3, VZ3.

10µl of sterile Z3 was added to the Z3 and VZ3 wells (25µM). 10µl of sterile DMSO was added to the B and DNV wells. 0.1 ml delta nef virus was added to the DNV and VZ3 wells above. Appropriate media was subsequently added to the wells in amounts to bring up the volume to 4ml per well.

Lastly, the culture plate was returned to the 37°C incubator, 10% CO_2.

On day 8, p24 antigen was assayed in culture supernatants as follows: With a micropippetor and sterile tip, 100µl of culture supernatant was removed from each set of H9 wells and transferred to an Eppendorf tube containing 20µl of a 10X solution of PBST (PBS+0.05% Tween 20). The Eppendorf tubes were capped and transferred to a 55°C water bath for 10 minutes. Subsequently, the Eppendorf tubes were returned to the sterile hood where p24 antigen levels were determined by the p24 Lateral Flow p24 antigen test in accordance with the manufacturer's product instructions. Results were recorded using BioRad Gel Doc System.

The medium was completely changed on day 8.

The H9 culture plate was transferred to the sterile hood and subsequently surface-wiped with70% EtOH. An appropriate number of 15 sterile culture tubes were collected under the sterile hood and labeled as indicated on the H9 culture wells. Using 5ml or 10ml sterile pipettes, cells were removed from the wells by repeatedly drawing cells into the pipette and transferring to the correspondingly labeled 15ml tube. Cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The tubes were returned to the sterile hood where medium was aspirated aseptically from the tubes. Using culture medium as above, cells were transferred to a new a 6-well cluster dish. DMSO-vehicle and Z3 inhibitor were added to appropriate wells as above. Medium was subsequently added to balance Z3 concentration at 25µM. Subsequently, the plate was returned to the 37°C incubator.

Cells were visually checked on days 10, 13, 17 and 21.

On day 22, the p24 assay described above was performed again in order to determine p24 antigen levels in culture supernatants. Results were recorded using BioRad Gel Doc system.

Results:

Figures 1A and 1B depict the results of the HIV-1 p24 antigen determination by Lateral Flow p24 antigen test on day 22 after exposure to Z3: Left to right, H9 cells (C), cells with inhibitor (Z3), Cells infected with HIV-1 (D-nef) and HIV-1 + Z3 (D-nef,Z3). The projected Z3 inhibition of endogenous HIV replication: > 90%.

Conclusion:

Based on the foregoing, the results reflect a clear inhibition of HIV replication in H9 cells upon administration of JAK2 kinase inhibitor Z3. Specifically, the results depict 90% inhibition of HIV-1 replication in Jurkat cells, thereby demonstrating that Z3 can be used to inhibit HIV-1 replication.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method for treating or preventing a viral disorder in a subject having or at risk of having said viral disorder, wherein said virus is RNA polymerase dependent, the method comprising administering to the subject an effective amount of a composition comprising a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby treating or preventing the viral disorder in said subject.

2. The method of claim 1, wherein the viral disorder is selected from the group consisting of HIV, influenzavirus, influenzavirus A, influenzavirus B, influenzavirus C, H1N1, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus.

3. A method for inhibiting the replication of a virus in a subject, comprising administering to the subject infected with said virus, wherein said virus is dependent on RNA polymerase for replication, an effective amount of a composition comprising a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby inhibiting viral replication in the subject.

4. The method of claim 3, wherein the virus is selected from the group consisting of HIV, influenzavirus, influenzavirus A, influenzavirus B, influenzavirus C, H1N1, hepatitis A, hepatitis C, hepatitis D, hepatitis E, and west nile virus.

5. The method of any one of the preceding claims, wherein the RNA polymerase is RNA polymerase II.

6. A method for treating or preventing HIV infection in a subject having or at risk of HIV infection, comprising administering to the subject an effective amount of a composition comprising a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby treating or preventing HIV in said subject.

7. A method for inhibiting the replication of HIV in a subject having or at risk of HIV infection, comprising administering to the subject an effective amount of a composition comprising a JAK2 kinase inhibitor or an agent that inhibits tyrosine phosphorylation of HEXIM1, thereby inhibiting HIV replication in the subject.

8. The method of any one of the preceding claims, comprising administering to the subject an effective amount of a JAK2 kinase inhibitor.

9. The method of any one of the preceding claims, wherein the JAK2 kinase inhibitor inhibits tyrosine kinase activity.

10. The method of any one of the preceding claims, wherein the JAK2 inhibitor is selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one, dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, or a combination thereof.

11. The method of any one of the preceding claims, wherein the JAK2 kinase inhibitor is AG490.

12. The method of any one of claims 1-10, wherein the JAK2 inhibitor is 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one.

13. The method of any one of claims 1-9, wherein the JAK2 kinase inhibitor is selected from the group consisting of low molecular weight inhibitors, antibodies or antibody fragments, peptide or RNA aptamers, antisense constructs, small inhibitory RNAs and ribozymes.

14. The method of claim 1-9, wherein the JAK2 kinase inhibitor is selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

15. The method of any one of the preceding claims, comprising administering to the subject an effective amount of an agent that inhibits tyrosine phosphorylation of HEXIM1.

16. The method of any one of claims 1-7 and 15, wherein the agent inhibits tyrosine phosphorylation of HEXIM1 at the YLEL domain of HEXIM1.

17. The method of any one claims 1-16, wherein the JAK2 kinase inhibitor or the agent that inhibits tyrosine phosphorylation of HEXIM1 is administered orally.

18. The method of any one of the preceding claims, further comprising monitoring the effectiveness of treatment.

19. The method of claim 18, comprising monitoring the activity of JAK2 kinase in the subject.

20. The method of claim 18, comprising monitoring the phosphorylation of HEXIM1 at the YLEL domain.

21. A method for treating or preventing HIV infection in a subject having or at risk of HIV infection, comprising administering to the subject an effective amount of an agent that inhibits phosphorylation of HEXIM1, thereby treating or preventing HIV in said subject.

22. The method of claim 21, wherein the agent inhibits JAK2 mediated phosphorylation of HEXIM1.

23. The method of claim 21 or 22, wherein the agent inhibits tyrosine phosphorylation of HEXIM1 at the YLEL motif of HEXIM1.

24. The method of any one of claims 21-23, wherein the agent that inhibits tyrosine phosphorylation of HEXIM1 is selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-

ylethyl)butan-1-one, dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, and 4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline.

25. The method of any one of claims 21-24, wherein the agent that inhibits tyrosine phosphorylation of HEXIM1 is a tyrphostin.

26. The method of claim 25, wherein the tyrphostin is AG490.

27. The method of any one of claims 21-24, wherein the agent that inhibits tyrosine phosphorylation of HEXIM1 is 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one.

28. The method of any one of claims 21-23, wherein the agent is an antisense nucleic acid molecule selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

29. The method of any one of claims 21-28, wherein the agent is administered orally.

30. A method for treating or preventing HIV infection in a subject having or at risk of HIV infection, comprising administering to the subject an effective amount of an agent that inhibits the release of HEXIM1 from a cyclin T1-cdk9 complex, thereby treating or preventing HIV in said subject.

31. The method of claim 30, wherein the agent that inhibits the release of HEXIM1 from a cyclin T1-cdk9 complex is selected from the group consisting of AG490, tyrphostin, lestaurtinib (CEP701), AZD1480, member of CIS/SOCS/Jab family, synthetic component AG490, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one, dimethoxyquinazoline compound, 4-(phenyl)-amino-6,7-dimethoxyquinazoline, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, 4-(3',5'-

dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline, or a combination thereof.

32. The method of claim 30 or 31, wherein the agent is a tyrphostin.

33. The method of claim 32, wherein the tyrphostin is AG490.

34. The method of claim 30 or 31, wherein the agent is 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one.

35. The method of claim 30 or 31, wherein the agent is an antisense nucleic acid molecule selected from the group consisting of an RNA interfering agent, an siRNA, an shRNA and dsRNA.

36. The method of any one of claims 30-35, wherein the agent is administered orally.

37. A method for identifying a therapeutic for treatment of HIV, comprising exposing a candidate substance to a composition comprising a JAK2 kinase or HEXIM1, monitoring the activity of the JAK2 kinase or HEXIM1, and identifying the candidate substance as a therapeutic for treatment of HIV if the candidate substance reduces the activity of the JAK2 kinase or the phosphorylation of HEXIM1.

38. The method of claim 37, comprising monitoring the tyrosine phosphorylation of HEXIM1, wherein a reduction in the phosphorylation of HEXIM1 identifies the candidate substance as a therapeutic for treatment of HIV.

39. The method of claim 37 or 38, comprising monitoring the JAK2 mediated phosphorylation of HEXIM1 at the YLEL motif of HEXIM1, wherein a reduction in the phosphorylation of HEXIM1 at the YXXL motif identifies the candidate substance as a therapeutic for treatment of HIV.

40. The method of any one of claims 37-40, further comprising monitoring the release of HEXIM1 from a cyclin T1-cdk9 complex.

Fig. 1A and 1B

A

One step lateral flow HIV p24 analysis. Arrow indicates the position of the p24 antigen on the test strip

B

Intensity of p24 bands in A was digitized and HIV p24 assigned a value of 100%.

Annexure - 3

The Challenge of Finding a Cure for HIV Infection

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Although combination therapy for HIV infection represents a triumph for modern medicine, chronic suppressive therapy is required to contain persistent infection in reservoirs such as latently infected CD4$^+$ lymphocytes and cells of the macrophage-monocyte lineage. Despite its success, chronic suppressive therapy is limited by its cost, the requirement of lifelong adherence, and the unknown effects of long-term treatment. This review discusses our current understanding of suppressive antiretroviral therapy, the latent viral reservoir, and the needs for and challenges of attacking this reservoir to achieve a cure.

Highly active antiretroviral therapy (HAART) for the chronic suppression of HIV replication has been the major accomplishment in HIV/AIDS medicine (*1*, *2*). Many patients are now in their second decade of treatment, with levels of plasma HIV RNA below the limits of detection of clinical assays. The impact on morbidity and mortality in the developed world has led to efforts that have brought this therapy to nearly three million people in resource-limited settings (*3*). Many patients are now enjoying a life-style little encumbered by symptoms or the side effects of medications, many of which require only once-daily administration. With the remarkable success of chronic suppression, why propose curing HIV infection—a challenging objective that requires potentially risky interventions and that may be unachievable?

Can We Do Better Than HAART?

HAART is no panacea. Current treatments must be maintained for life, with treatment interruption resulting in the rapid rebound of replicating virus. Although drug resistance can emerge because of the challenges of maintaining adherence and access to chronic antiviral therapy or owing to transmitted drug-resistant viruses, the success of HAART has been improved by the development of more potent and more tolerable therapies. Successful new drug development may not continue indefinitely, however, and HAART may never reach the majority of infected individuals in less-developed countries. Despite the prolonged suppression of HIV replication below the standard limits of detection for patients on HAART, ongoing viremia can be detected at levels of 1 to 50 copies per milliliter in the majority of patients (*4*, *5*). The origin of this viremia has not been fully characterized, but it does not appear to jeopardize the prolonged success of therapy in the adherent patient (*6*). Nevertheless, the virions may engage CD4 and chemokine receptors and may activate pathways that could lead to chronic consequences, including cardiovascular and malignant disease. The suboptimal penetration of many antiretrovirals into the central nervous system may also permit low levels of viral replication and/or release from stable viral reservoirs, resulting in neuropathology (*7*, *8*).

Despite the very low rates of toxicity of many of the newer HAART regimens, many of these drugs modulate lipid and glucose metabolism (*9*). Even modest toxicities may have cumulative effects over decades of treatment. Moreover, prolonged treatment may reveal toxicities not appreciable with animal toxicology or several years of clinical surveillance. There is already growing concern about increased rates of heart disease, diabetes, liver disease, and many forms of cancer in aging HIV-infected patients who are receiving treatment (*10*–*13*). Whether these are because of long-term HIV infection, therapeutic drug treatment, or both, is uncertain. Finally, the cost of HAART may be too much to sustain treatments on a global scale, as millions are affected.

Given the shortcomings of HAART, time-limited interventions that do not result in the resumption of viremia are a desirable but a currently unattainable objective, unlike what can be achieved with the treatment of hepatitis C virus infection. Such therapy might or might not eliminate every functional virion or infected cell, but would permit the discontinuation of HAART without the reappearance of viremia and disease. We propose that a drug-free remission should be the new goal of HIV therapeutics.

What Is the State of HIV in Successfully Treated Patients?

The source of the low-level viremia seen in most patients on HAART (*4*, *14*, *15*) may be incompletely characterized, but we do have some hints (Table 1). The failure, thus far, of treatment intensification to clear this viremia (*16*) and the lack of evidence for nucleotide sequence evolution over long periods of treatment (*17*–*19*) indicate that this phenomenon may not be driven by ongoing rounds of replication.

Patient data reveal that 1 in 10^6 CD4$^+$ T cells are latently infected with HIV, despite the durable suppression of detectable plasma viremia, although the frequency can be much lower in some patients (*20*–*22*). In vivo, it is thought that these cells are intermittently activated by antigen recognition or as bystanders in a local inflammatory process, which leads to the release of progeny virions.

Another source of virion production, which does not require ongoing replication, is the episodic production of HIV by long-lived cells. In situ hybridization of lymphoid tissue in simian immunodeficiency virus (SIV)–infected macaques and HIV-infected humans revealed that, in addition to the activated and infected CD4$^+$ T cells that produce large numbers of virions with a short cellular half-life, many lymphocytes can be visualized that produce small amounts of viral RNA, yet do not display markers of activation (*23*). Such cells are not seen in vitro, and whether such cells occur in vivo during prolonged antiretro-

Table 1. HIV latency.

- Latently infected resting memory CD4$^+$ T cells are the best-characterized latent reservoir for HIV-1.
- Less than 1 cell per 1,000,000 resting CD4$^+$ T cells from patients on HAART harbor latent HIV-1 provirus.
- Sequence of latent proviruses does not evolve, which suggests no ongoing viral replication.
- Discontinuation of HAART allows viral relapse from latent reservoir.
- Patients successfully treated with HAART for longer than 10 years exhibit no appreciable decrease in the size of the latent reservoir.
- The persistence of latently infected memory CD4$^+$ T lymphocytes precludes their elimination by HAART alone for the lifetime of the patient.
- Other drug-insensitive reservoirs, including brain, macrophages, and hematopoietic stem cells, may also exist.
- Latency is likely established and maintained by numerous blocks at multiple steps in the HIV-1 replicative pathway, which potentially complicates eradication strategies.

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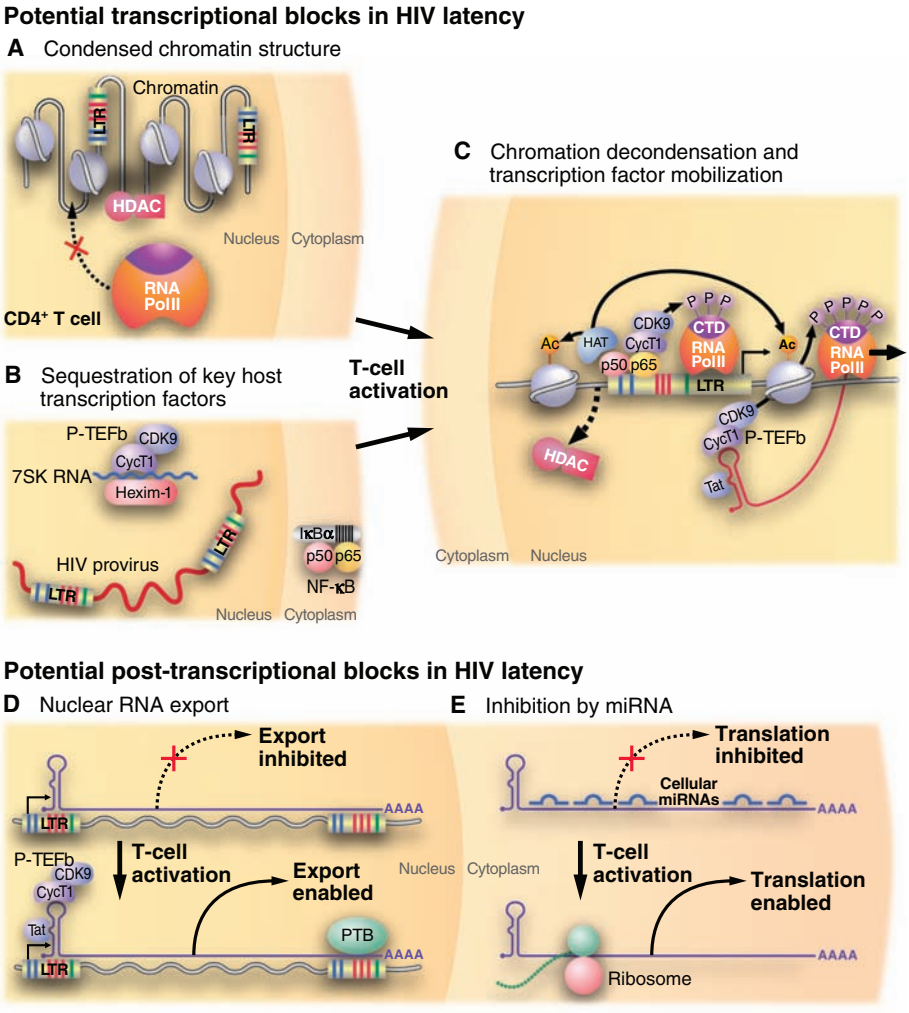

Fig. 1. Proviral latency is the result of multiple restrictions on HIV expression. (**A**) Proviral latency is maintained, in part, by the action of several transcription factors that recruit HDACs and other complexes to the HIV-1 long-terminal repeat (LTR) promoter, which results in histone modifications within chromatin at the HIV promoter that limit the ability of RNA polymerase to initiate transcription. (**B**) Key cellular factors that are required for robust HIV transcription, such as NF-κB or the P-TEFb–cyclin complex, are sequestered in resting $CD4^+$ T cells by cellular regulatory complexes [inhibitor of nuclear factor κB (IκB) and HEXIM–7SK RNA, respectively). Release and mobilization of these factors is required for proviral expression. (**C**) When histone acetyltransferases (HATs) supercede the effect of HDACs, coactivators such as NF-κB can recruit RNA polymerase (RNA Pol) complexes. Production of Tat allows the recruitment of P-TEFb, mediating an explosive increase in transcription and the escape of provirus from latency. (**D**) The initial wave of Tat production may be further restricted by inefficient export of multiple spliced HIV mRNAs, relieved upon cellular activation by enhanced expression of PTB. (**E**) Cellular miRNAs that bind HIV mRNAs may also restrict translation of early expressed HIV mRNAs and so reduce Tat production. CDK, cyclin-dependent kinase; CTD, C-terminal repeat domain; and CycT1, cyclin T1.

viral therapy is unknown. Further, the life span of and the kinetics of viral expression in such cells remain undefined.

Low-level plasma viremia cannot always be linked to activation of latently infected $CD4^+$ T cells. In a longitudinal analysis of cloned RNA from plasma-derived virions of a subset of HAART-suppressed patients, the Siliciano group identified distinctive homogeneous viral subpopulations (*24*). These observations raise the possibility of a chronically infected clonal reservoir, analogous to a persistently infected stem cell. How a persistently infected cell population could produce virions at a steady state for years, in the presence of some level of cell-mediated immunity, remains unexplained. Other cellular or tissue sources of virus, such as cells of the monocyte and macrophage lineages, may also contribute to low levels of viremia.

Can Mechanisms That Drive Latency Be Therapeutically Exploited?

Activation from latency to completion of the replication cycle should result in lytic cell death of $CD4^+$ T cells. Multiple mechanisms may contribute to the maintenance of proviral latency [reviewed in Williams and Greene (*25*)], and so, combination approaches could be required to eradicate infection (Fig. 1 and 2). Such strategies would depend on current or future antiretroviral therapy to completely inhibit all new infection events. Antilatency agents would be given, intermittently and for a limited period of time, to purge the last sanctuaries of HIV infection (Fig. 3).

Chromatin remodeling enzymes like histone deacetylases (HDACs) play a critical role in HIV latency (Fig. 1A) (*26*–*29*). HDACs are recruited to the highly conserved initiator region of the HIV promoter by several distinct complexes, by means of factors that are both ubiquitous in cell types infected by HIV and also participate in basal and activated viral gene expression. The existence of multiple mechanisms that recruit repressive HDAC complexes to the proviral promoter raises the possibility that HDAC inhibitors might lead to the activation of HIV in latently infected cells (Fig. 2).

In addition to HDACs, HIV expression is limited by other cellular barriers to effective mRNA transcription, which the virus overcomes through the action of its own activator, Tat. Tat recruits the positive transcription elongation factor b (P-TEFb) kinase to the integrated viral promoter, inducing viral gene expression (Fig. 1B and C) (*30*). Several kinase agonists, including hexamethylbisacetamide (HMBA)—a compound previously tested in human cancer trials (*31*), activate intracellular signaling cascades that mobilize P-TEFb in the absence of Tat (*32*, *33*) and can induce the expression of HIV in latently infected cells (Fig. 2) (*34*).

The HIV promoter responds to coactivators that are abundant in activated cells, but, in the context of the resting T cell, inadequate nuclear levels of nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT) may contribute to the establishment of latency (Fig. 1B) (*35*). Diminished binding could be the result of changes in chromatin structure, in part mediated by the action of HDACs. Prostratin, a nontumorigenic phorbol ester isolated from the Samoan medicinal plant, *Homalanthus nutans*, induces HIV expression in latently infected cell lines and cells isolated from HIV-infected, HAART-treated patients in the absence of cellular proliferation (*36*). In cell-line models, prostratin stimulates HIV expression through protein kinase C–mediated activation of NF-κB and so provides an approach to activation and clearance of latently infected cells (Fig. 2) (*37*).

HIV mRNA export may also be impaired in resting T cells because of the low levels of polypyrimidine tract–binding protein (PTB) available in resting cells (Fig. 1D) (*38*). MicroRNAs (miRNAs) endogenously expressed in human cells may further impede HIV mRNA expression or translation (Fig. 1E) (*39*, *40*). If such mechanisms contribute to proviral persistence, entirely new classes of therapeutic agents able to safely alter host RNA expression or transport will be required.

Given the intimacy of the interaction between the retrovirus and the host cell, therapeutic approaches that disrupt latent infection are also likely to affect host cell function. Although mild host toxicities for limited periods of time might be acceptable, global immune activation must be avoided. Once quiescent virus is successfully induced to complete a round of replication, virus-induced cytolysis and cytotoxic T cells need to be able to clear HIV antigen–expressing cells. The viral progeny generated by such activated cells have to be prevented from successfully infecting other cells by the presence of HAART (Fig. 2).

How Are Interventions to Be Investigated?

Undoubtedly, there are other factors that regulate latency occurring in primary cells in vivo. Although we need to be aware of the potential for additional reservoirs of infectious virus, addressing the latently infected T cell reservoir may be the most direct way of exposing an even smaller additional reservoir, like infected macrophages, or anatomic compartments, such as the central nervous system, that may be suboptimally exposed to HAART. Careful in vivo testing of therapeutic agents capable of antagonizing the different mechanisms underlying HIV latency identified in $CD4^+$ T cells is important for establishing the proof of concept.

An animal model is not required for antiretroviral drug development because, thus far, activity in vitro has correlated with activity in vivo. In contrast, an animal model could be invaluable in the development and testing of antilatency therapies and would guide clinical trial design. Given the excellent outcomes of HAART, initial studies of new antilatency therapies in humans might be difficult to design and execute, because volunteers in such early studies may have little to gain, and the candidate interventions will have unproven efficacies and uncertain toxicities. SIV infection in the rhesus macaque gives rise to latent infections in $CD4^+$ T cells that mirror HIV latency (*41*), although it remains unknown whether the pathways and molecular targets promoting postintegration latency in macaques are the same as in humans.

BLT (bone marrow-liver-thymus) mice provide a second animal model. These immunodeficient mice (which lack endogenous T and B cells) are transplanted with human thymus and liver tissue and injected with hematopoietic stem cells, giving rise to systemic repopulation with human T and B cells, monocytes-macrophages, and dendritic cells capable of antibody production, activation by human antigen-presenting cells, and potent human major histocompatibility complex–restricted T cell immune responses (*42*). BLT mice have already been used to study HIV transmission and to test preexposure antiretroviral prophylaxis (*43*). Determining whether this model can be used to study HIV latency is a high experimental priority. Despite the availability of animal models for preliminary testing, clinical studies in HIV-infected patients are ultimately required.

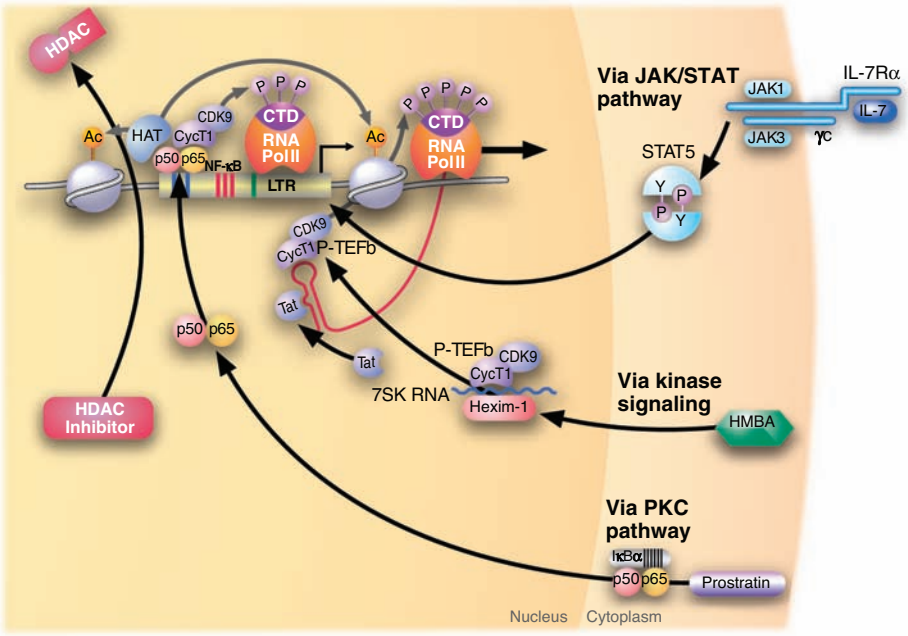

Fig. 2. Potential therapies to disrupt latent proviral HIV infection. HDAC inhibitors may relieve repression by HDACs and may allow histone acetylation by HAT, which results in HIV expression. Via kinase signaling, HMBA stimulates the release of P-TEFb from sequestration within a ribonucleoprotein complex containing HEXIM and 7SK snRNA (small nuclear RNA). Tat then recruits P-TEFb to an HIV RNA structure (TAR), present at the 5′ end of all nascent HIV RNAs, which allows for phosphorylation and activation of RNA Pol II and other factors, leading to processive transcription. Prostratin stimulates HIV through protein kinase C (PKC)–mediated release of active NF-κB. Interleukin 7 (IL-7), a cytokine essential for maintenance of T cell homeostasis, can induce HIV expression from quiescent resting cells without global T cell activation, via the JAK/STAT5 signaling pathway.

Phase I trials to deplete persistent HIV infection have demonstrated that these approaches can be tested safely (*44*–*46*), and studies using novel inducers of HIV expression such as interleukin 7 (*47*) may soon be feasible (Figs. 2 and 3).

Quantifying the latent HIV reservoir in humans is challenging when less than 1 in a million $CD4^+$ T cells are latently infected, and there are approximately 100 copies of integrated provirus for each latently infected $CD4^+$ T cell (*48*). After amplification by the polymerase chain reaction, measurements of integrated proviral DNA might serve as a surrogate marker for changes in the latent reservoir (*18*). However, the small size of the reservoir and the imprecision of current assays require improved techniques to assess the effectiveness of interventions. Moreover, once the reservoir is reduced by 10- to 100-fold, the remaining latently infected cells may be concealed below the limit of detection of any assay yet described.

Access to lymphoid tissue or most anatomic compartments in otherwise healthy subjects is difficult. Although such studies may fail to detect an infected reservoir, they cannot prove its eradication. When an intervention or combination of interventions is considered sufficiently compelling, the ultimate test of efficacy will be the withdrawal of HAART. Antiretroviral therapy is effective and relatively safe. As a result, the administration of any experimental intervention in either a proof-of-concept feasibility trial or in a trial incorporating treatment interruption raises significant ethical, regulatory and study design issues, because antiretroviral therapy is so effective and relatively safe. Therefore, involvement of various stakeholders in thoughtful deliberations is necessary. Such studies are required if we wish to cure HIV; but, although the potential benefit to humanity is great, the benefit to the early trial volunteers is nearly nonexistent. The appropriate volunteers in a trial involving treatment interruption might be those who initiated HAART before significant immune depletion. This criterion would minimize risk of treatment interruption, especially with close monitoring to resume treatment should virus replication be detected. A second rationale for selecting such subjects is that their infected-cell reservoir may be smaller and thus more amenable to intervention (*18*, *49*).

Do We Need a New Approach to Develop a Cure?

The recent disappointing results from the trials of HIV vaccine and microbicide candidates have prompted a renewed commitment to basic research to identify effective approaches to these critically needed prevention strategies. We advocate a similar impetus for new approaches to purge the latent reservoir in order to cure HIV infection.

Years of effort have led to public health strategies to reduce the risk of cancer, a vaccine

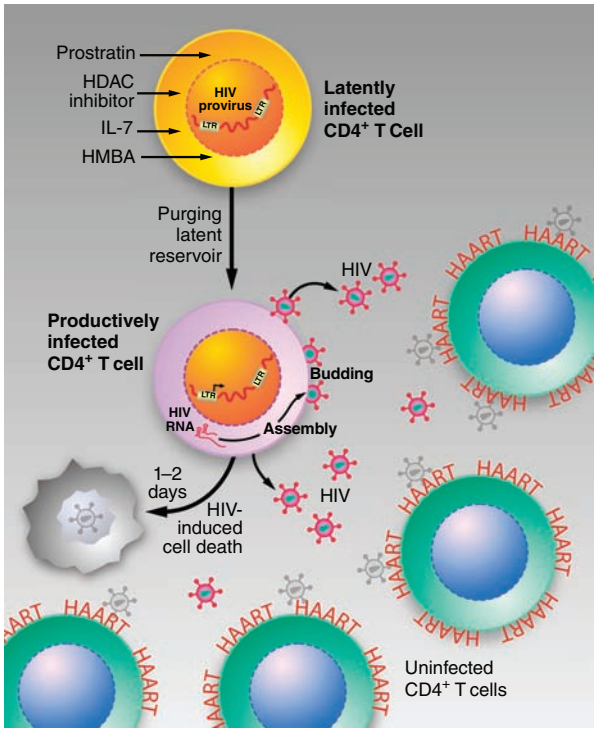

Fig. 3. Purging persistent proviral infection. If targeted approaches, alone or in combination, succeed in activating latent HIV proviruses present in differentiated CD4+ T cells, the life span of these cells should be short. These inductive agents must be used in combination with HAART to prevent further HIV spread to uninfected CD4+ T cells.

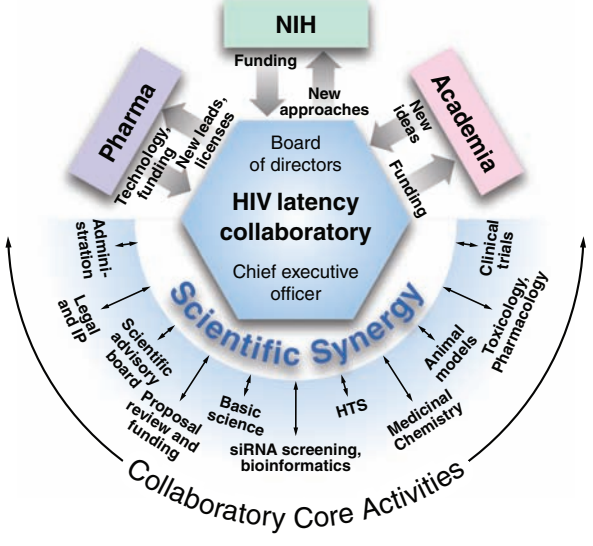

Fig. 4. Overview of an HIV Latency Collaboratory representing a joint research venture between the U.S. National Institutes of Health (NIH), the pharmaceutical industry (pharma), and academia. The goal of this collaboratory is to accelerate basic discovery and the clinical translation of these discoveries that allows for eradication of HIV in infected patients or at least a stable drug-free remission. This collaboratory is designed to include all interested investigators with meritorious ideas. These investigators will be funded via the collaboratory and will have access to a range of core technologies provided by the collaboratory that should promote scientific synergy and enhanced experimental efficiency. The collaboratory would be governed by a Board of Directors comprised of representatives from each of the partners in the joint venture. HTS, high-throughput screening; IP, intellectual property; and siRNA, small interfering RNA.

that prevents cervical cancer, better therapies to treat malignancies, and curative therapies for some cancers. Such a multifaceted approach should also be applied to the effort to cure HIV infection. This will require behavioral and biological tools to prevent HIV infection; safe, affordable, and nontoxic therapies for initial control of HIV infection; and new interventions that can achieve a drug-free remission of viremia in some patients.

The challenge of developing an HIV vaccine spans the need for new basic research insights to product development to clinical trials. The complexity of fostering and coordinating these efforts has led to the creation of major NIH intramural (Vaccine Research Center) and extramural (Center for HIV/AIDS Immunology) programs and of an international, multi-institutional effort (The Global HIV Vaccine Enterprise). Our understanding of HIV latency has chiefly resulted from independent, investigator-initiated efforts. In order to translate these academic accomplishments into clinical treatments similar initiatives are required. Antilatency therapies will require the drug discovery capabilities of industry, like high-throughput drug candidate screening; medicinal chemistry; product synthesis, production, and formulation; toxicology; and pharmacology. A coordinated initiative involving academia, industry, government, and patient advocates could greatly accelerate the identification of potential interventions and their clinical assessment (Fig. 4). We conceive an initiative, termed here a collaboratory, in which the government contributes funding, regulatory oversight, and coordination; industry contributes funding, drug discovery, technology, and expertise; and academia contributes ideas and investigative capacity. Long-term support for a flexible, collaborative public-private joint venture might improve efficiency and conserve resources, while at the same time catalyzing progress that no single group could achieve. Clearly much work and many challenges lie ahead, but if novel scientific insights can be brought to bear in clinically effective ways, the era marked by the benefits of HAART may be followed by one in which HAART is no longer a lifelong necessity.

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Mechanisms of HIV Latency: an Emerging Picture of Complexity

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Abstract Rarely HIV type 1 establishes proviral latency within the host genome, maintained with little or no viral gene expression. This state has been quantitated in peripheral blood and lymphoid tissues of HIV-infected patients, appearing in the earliest days of infection. These rare cellular reservoirs are unaffected by current antiretroviral therapy and unrecognized by the host immune response, and can regenerate disseminated viremia if therapy is interrupted. Proviral latency may be established when a newly HIV-infected cell exits the cell cycle and returns to the resting state. Rarely, direct infection of resting cells may also occur. Multiple molecular mechanisms appear to underlie the establishment and maintenance of persistent, latent HIV infection, most frequent in the resting central memory CD4+ T cell. Interrupting processes that maintain latency may allow therapeutic attack of this primary form of persistent HIV infection, but a better understanding of relevant mechanisms in vivo is needed.

Keywords Latency · Resting CD4+ T cell · Chromatin · Histone · HDAC · Methylation · IL-7

Introduction

Antiretroviral therapy (ART) has markedly decreased morbidity and mortality in HIV-1-infected individuals in the developed world. Successful therapy often results in stable plasma levels of HIV-1 RNA below the limits of detection of clinical assays. Nevertheless, HIV-1 infection has not been cured by ART. The causes of persistence of HIV infection in the face of current therapy appear to be multifactorial: latent but replication-competent provirus in resting $CD4^+$ T lymphocytes, cryptic viral expression below the limits of detection of clinical assays, and viral sanctuary sites might all contribute to persistence.

Evidence that cellular factors are required to maintain quiescence implies that proviral latency is an unstable state of HIV infection, amenable to therapeutic attack. An understanding of how latency is maintained may lead to novel therapies directed at the viral reservoir within resting cells. Other obstacles to the therapeutic clearance of established HIV infection exist—therapies to attack proviral genomes must be developed before eradication of HIV infection can be considered.

Clearance of HIV infection will almost certainly require a multimodality approach that includes potent suppression of HIV replication, therapies that reach all compartments of residual HIV replication, and depletion of any reservoirs of persistent, quiescent proviral infection. Here we highlight the basic mechanisms for the establishment and maintenance of viral reservoirs, suggesting approaches toward their elimination.

The persistence of virus in HIV-infected patients receiving potent ART was conclusively demonstrated when rare, integrated, replication-competent HIV was recovered from patients' resting CD4 memory T cells [1–3]. To date, this reservoir remains the most widely studied and best understood cause of viral persistence. Evidence suggests that the resting T-cell reservoir is established early after infection and is extremely stable. Current estimates based on long-term clinical studies suggest that the half-life of these stably infected cells is approximately 44 months in successfully treated individuals [4], and that without ART

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viremia reemerges from these reservoirs [5]. The eradication of infection by current ART is thus impractical.

It cannot be overemphasized that the persistence of HIV infection despite ART is not a unidimensional problem. Low-level replication of HIV may persist in other cell types or distinct sequestered compartments, such as the gut-associated lymphoid tissue or central nervous system. Persistent expression of HIV RNA (without evidence of full rounds of replication) can be detected in HIV-infected patients on durably successful ART by research assays in the plasma [6]. It is incompletely understood how expression may persist on ART without the development of drug resistance; one explanation is that persistent expression of HIV RNA originates exclusively in cells that were infected prior to ART initiation.

Nevertheless, as antiretroviral drugs improve, allowing increasingly potent inhibition of active HIV-1 replication, a role for therapeutic tools to attack the persistent proviral state is emerging. This is illustrated by the significant increase in the level of effort directed around the world to understanding the mechanisms that underlie HIV proviral latency, and the number of significant insights gained recently. These advances give hope that rational therapeutic approaches to the persistent proviral state might reduce the frequency of the emergence of drug resistance, improve the ability of an augmented immune response to contain HIV replication, or ultimately allow the clearance of HIV infection.

Mechanisms That Allow Persistent, Latent HIV Infection

The prevailing hypothesis for the establishment of latently infected CD4+ T cells in patients is that the virus infects T cells just prior to their natural reversion to a quiescent state, as with memory T cells, or in the case of the naïve T-cell population, infects cells that are undergoing differentiation during thymopoiesis. Given the potency of the viral activator Tat, and the responsiveness of the HIV promoter to many cellular activating signals, counterregulatory mechanisms that repress transcription appear to be required to allow HIV to establish or maintain a persistent, nonproductive infection. Although expression of the HIV-1 long terminal repeat (LTR) promoter is augmented by numerous cellular factors, relatively few factors have been identified that downregulate the LTR [7].

Deficiency of Host Factors Required for HIV Expression

Several features of the metabolism of resting CD4 cells are critical for the establishment of latency [8]. First, resting cells lack the coactivating factors nuclear factor (NF)-κB or nuclear factor of activated T cells. Induction of either transcription factor by drugs, or by T-cell receptor activation, provides a powerful signal leading to the resumption of transcription by latent HIV proviruses. Critically, the viral transactivator, Tat, recruits a transcription complex that contains novel components, including the transcription elongation factor P-TEFb (positive transcription elongation factor b). Reductions in the level of the P-TEFb component CycT1 and sequestration of the P-TEFb complex by the HEXIM/7SK RNA complex also appear to restrict HIV transcription in resting lymphocytes. In addition to these mechanisms, it has also been suggested that HIV mRNA export is impaired in resting T cells, posing a further barrier to expression of provirus in resting cells [9]. There is evidence that host microRNAs can impede HIV mRNA expression or translation [10, 11].

Transcriptional Interference

Recent data have demonstrated that transcriptional interference mediated by nearby host gene promoters contributes to the quiescence of some HIV proviral genomes [12, 13]. This concept arose following the observation that most viral integrants reside in introns of actively transcribed genes [14]. Therefore, host gene "readthrough" transcription could prevent transcription initiation within the HIV LTR viral promoter. This effect could be negative if host and viral genes are convergently transcribed, but could prevent latency and upregulate proviral expression if the provirus is integrated in the same orientation as the host gene. Using two different model systems, Han et al. [12] made observations consistent with these expectations, but Lenasi et al. [13] discovered that convergent transcription inhibited downstream HIV expression, presumably by promoter occlusion, and that inhibiting upstream expression in this situation allowed HIV expression. The findings illustrate the complexity of eukaryotic transcriptional systems. Indeed, both situations could exist if the effect of transcriptional interference differs depending on the distance between the interacting promoters. Nevertheless, if an integrating provirus lands in a genomic location and orientation favorable for negative transcriptional interference, this integrant may be much more likely to establish latency. However, given the multiplicity of integration sites, each of potentially different orientation and spacing in a spectrum of host promoters, it is difficult to envision a translational strategy to deplete persistent infection that takes advantage of this variegated regulatory mechanism.

Chromatin Restrictions

Histone Acetylation

Transcription factors have long been known to affect chromatin structure, regulating gene expression, and may

influence proviral latency of HIV (Fig. 1). Multiple signaling pathways result in enzymatic covalent modifications (eg, acetylation, phosphorylation, and methylation) of specific amino acids in histone tail domains. The "histone code" hypothesis holds that combinations of distinct modifications occurring at particular sites on the histone tail direct which proteins are capable of interacting with histone–DNA complexes and determine gene activity [15]. Already more than 50 enzymes are known that selectively modify the histone tail, thus providing the means to make a combinatorial histone code. One canonical example is that of the histone acetylases, acting to allow the transcriptional machinery access to the DNA template, compete with histone deacetylases (HDACs) that blunt transcription by reducing accessibility of DNA templates. These modifications do not simply make chromatin more or less accessible, but inscribe biophysical marks on gene regions, signals for the ordered recruitment of complexes of regulatory factors that up- or down-regulate gene expression. There is a significant body of evidence suggesting that these mechanisms regulate HIV expression as well.

Important work from the Verdin laboratory has shown that transcription is restricted by the presence of a strictly positioned nucleosome (Nuc-1) that is very close to the viral RNA start site (+10 to +155). In models of chronic HIV infection, increased accessibility of chromatin about the LTR has been associated with transcriptional activation [16]. More recently, it has been shown that reactivation of HIV transcription requires remodeling of the critical Nuc-1 by SWI/SNF [7, 8].

It was originally believed that integration into the quiescent or centromeric region of the human genome, or acquired mutations of the virus itself [17], were responsible for the creation of these chromatin structures. However, evidence from both laboratory studies and more recent studies of resting CD4+ T-cell populations obtained from HIV-infected patients shows that HIV integrates preferentially into active regions of the human genome [18]. It therefore seems more likely that epigenetic silencing contributes to the entry of HIV into latency, given what is known about the suppression of inappropriate initiation sites mediated by transcription-directed histone modifications.

The first example of a host silencing mechanism that downregulates HIV expression was that of the cooperative recruitment of HDAC1 to the HIV LTR by the host transcription factors YY1 and LSF [19]. Synthetic small molecules designed to specifically inhibit LSF binding to the LTR and HDAC1 recruitment induced the outgrowth of HIV from resting CD4+ T cells obtained from HIV-infected donors, demonstrating that specific and targeted inhibition of HDAC activity at the HIV LTR disrupts proviral latency in HIV-infected patients' cells [20]. Consistent with a major role for HDACs in establishing HIV latency, many drugs that inhibit HDAC activity, such as trichostatin A (TSA) and valproic acid (VPA) [21], are effective inducers of HIV transcription in latently infected cells.

HDACs are unable to directly associate with proviral DNA but are recruited through cellular DNA-binding proteins that recognize sequences in the viral LTR, including YY1, NF-κB p50, and AP-4. A fourth complex containing c-Myc and Sp1 was found to recruit HDAC1 to the LTR. Recently, CBF-1, a CSL (CBF1/RBP-Jkappa/suppressor of hairless/LAG-1) type transcription factor and key effector of the Notch signaling pathway, was shown to be a remarkably potent and specific inhibitor of the HIV-1 LTR promoter, which acts by recruiting HDACs [22]. These findings echo the ability of multiple coactivator complexes to recruit histone acetylases to promoters, including the HIV LTR [23].

To identify all the potential HDACs involved in HIV transcription regulation, we recently performed a systematic survey for the presence of HDACs, in addition to HDAC1, at the LTR [24•]. We found RNA expression for all 11 isoforms of HDACs (including four splice variants of HDAC9) in the resting CD4+ T cells of HIV-infected, aviremic, ART-treated patients. The highest expression levels were seen for HDACs 1, 3, and 7, intermediate expression was seen for HDACs 2, 4, and 5, and HDACs 6, 8, 9, 10, and 11 showed low expression levels. Similarly, HDAC protein was detected by Western blot in whole cell extracts of these cells, except for HDACs 9 and 10. Overall, the class I HDACs 1, 2, and 3 and the class II HDACs 4, 6, and 7 could be detected by Western blot in the nucleus of resting CD4+ T cells and are therefore candidate enzymes for direct regulation of integrated HIV genomes.

Chromatin immunoprecipitation (ChIP) can directly document the association of factors at specific promoter regions and therefore could provide definitive proof of local regulation of the HIV LTR by HDACs. However, it is not possible to perform the ChIP assay in patients' cells, as the target viral DNA is too rare. Therefore, we used a Jurkat CD4+ T-cell model system to study HDAC occupancy at the HIV LTR. In cells verified to encode a single HIV LTR provirus, RNA and protein for HDACs 1 to 11 are easily detectable in the cytoplasm and/or nucleus. However, occupancy of only the class I HDACs 1, 2, and 3 is detectable by ChIP at the HIV LTR using the same antibodies that detect these proteins by Western blot in J89s and primary cells [24•]. The expected loss of HDAC occupancy after TSA exposure provides further validation of the specificity of these ChIP results. Changes in ChIP occupancy seen for HDAC1 and 2 are statistically significant ($P<0.05$) by real-time DNA quantitation. Occupancy of the LTR by class II HDACs (ie, HDACs 4–7, 9, and 10) was not observed.

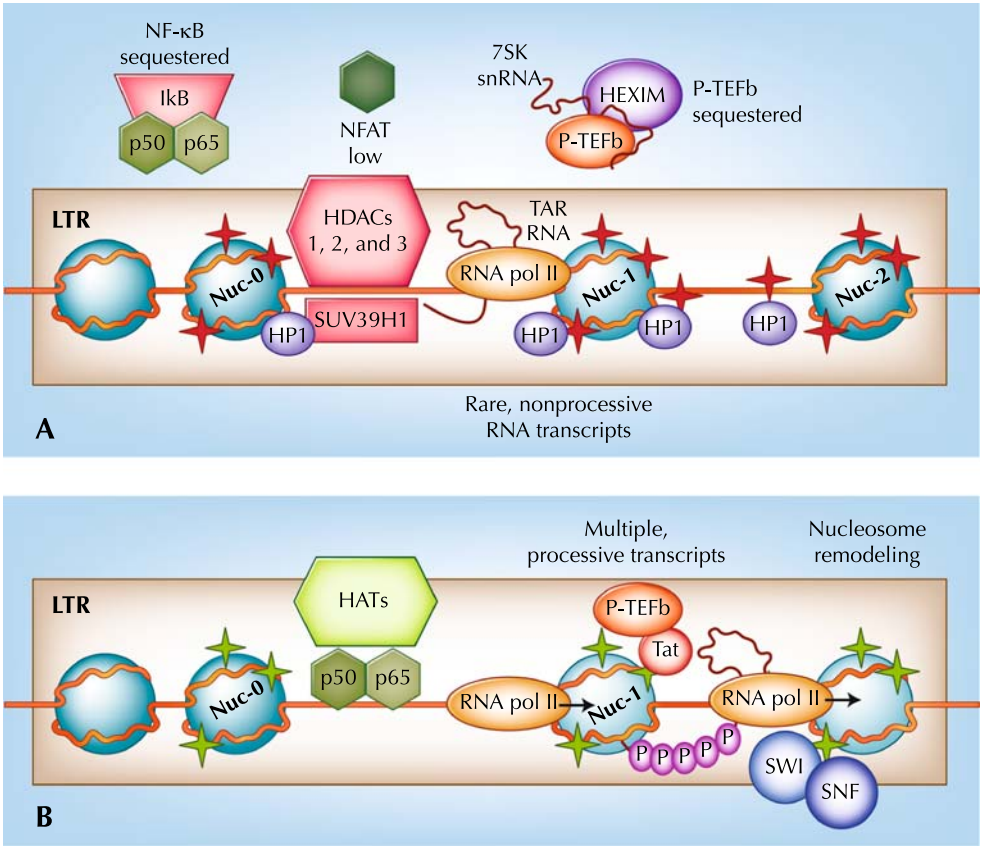

Fig. 1 Molecular mechanisms that allow or restrict HIV proviral expression in quiescent T cells. **a** At the HIV long terminal repeat (LTR), histone deacetylases (HDACs) 1, 2, and 3 lead to histone deacetylation (*pink*), and this allows recruitment of histone methyltransferases such as SUV39H1, and heterochromatin proteins such as HP1-α. Methyltransferases can ultimately methylate DNA (*red*). In this setting some polymerase complexes may initiate transcription, but infrequently due to low nuclear levels of co-activators such as nuclear factor (NF)-κB and nuclear factor of activated T cells (NFAT). Transcription that is initiated is abortive and nonprocessive due to the lack of positive transcription elongation factor b (p-TEFb). **b** When NF-κB or NFAT are available, histone acetyltransferases (HATs) can be recruited. HATs acetylate chromatin, assisting in transcription complex recruitment. As transcription proceeds, nucleosome remodeling complexes such as SWI/SNF enhance the opportunity for subsequent waves of transcription. Early waves of transcription produce the HIV transactivator protein Tat, which then actively recruits the p-TEFb complex to the TAR RNA structure at the 5′ end of the nascent HIV RNA. This allows for phosphorylation and activation of RNA polymerase (pol) II and other factors, leading to an explosive increase in transcriptional activation and the escape of provirus from latency

To explore the specific roles of individual class I HDACs, siRNA knockdown studies targeting single class 1 HDACs were performed in a reporter cell line. siRNAs targeting the class I HDACs 1, 2, 3, and 8 or nonspecific siRNAs were tested. siRNA-mediated knockdown of HDAC2 induced a significant ($P<0.001$) increase in LTR-driven expression compared with the mock control. However, as siRNA is usually incomplete, even with multiple siRNAs (as in these experiments), we repeated these studies in the presence of global HDAC inhibition (TSA), increasing the basal level of HIV LTR expression approximately 20%. In the presence of submaximal HDAC inhibition, siRNA knockdown of HDAC3 upregulated LTR-driven expression. Similar studies utilizing primary cells rather than cell lines will be required to validate the specific roles of class I HDACs in HIV latency.

In companion studies [25•], we tested the effect of selective HDAC inhibitors (HDACi) at concentrations that induce HIV LTR expression in cell line models. Findings illustrate the mean frequency of viral recovery in the presence of maximal mitogen or HDACi. Nonselective (VPA) and class 1-selective HDACi allow the recovery of virus from patients' resting CD4+ T cells. Effects on cell viability do not account for this difference. Paradoxically, HDACi that are highly selective for the principal HDACs that are resident and active at the LTR, the class I HDACs 1, 2, and 3, are optimal inducers of viral outgrowth, although they are sufficient for proviral expression. Toxicity or inhibition of cell growth is not significantly different at the concentrations used in these studies. There is more to be learned about the mechanisms of HIV repression mediated by HDACs and the optimal use of HDACi.

Histone Methylation

In addition to regulation of histone acetylation at the latent provirus, two recent reports demonstrate that the histone methyltransferase Suv39H1, histone H3 methylated on K9, and the repressive HP1 proteins all accumulate on transcriptionally inactive proviruses [26, 27]. Finally, in an important and elegant study, Pearson et al. [28•] demonstrated that progressive, iterative histone modifications can drive a proviral promoter into latency within primary CD4+ T cells.

Methylation of DNA itself is an important and durable epigenetic mark, but the role of DNA methylation in HIV latency has been controversial. Recently, Blazkova et al. [29•] and Kauder et al. [30•] presented new evidence that CpG methylation of HIV promoter DNA can durably suppress HIV expression. Ironically, it was the potent HDACi suberoylanilide hydroxamic acid that was demonstrated to most efficiently reactivate densely methylated HIV-1 promoters.

Persistence Via Homeostasis

A totally different model of persistent infection within resting CD4+ T-cell infection has recently been put forward. It implies that proviral infection is not completely stable, and that in fact infected resting cells leave the quiescent memory pool, but the frequency of infection in this pool of cells is maintained by homeostatic proliferation of infected cells.

Chomont and colleagues [31••] recently reported that central (T_{CM}) and transitional memory (T_{TM}) CD4+ T cells are the major cellular reservoirs in which integrated HIV DNA persists in patients. Their data suggest that viral persistence is maintained through T-cell survival and low-level antigen-driven proliferation and is slowly depleted with time in T_{CM} cells. In contrast, in aviremic patients with lower CD4+ counts and higher levels of interleukin (IL)-7-mediated homeostatic proliferation, proviral DNA is preferentially detected in T_{TM} cells. This finding suggests that IL-7-driven proliferation may result in host-driven replication of proviral genomes without the death of these infected cells, ensuring the persistence of this reservoir. Although the numbers of cells available were too limited to perform robust quantitation of replication-competent virus with memory cell populations, recovery of virus in co-culture assays was generally consistent with integration frequency by *Alu*–polymerase chain reaction. Patients with immune preservation (higher CD4 counts) had proportionally more central memory and naïve cells, and fewer proviral integrants, and patients with lower CD4 counts had proportionally more proviral integrants found in effector memory T cell populations.

Conclusions

Chronic, lifelong ART may be needed for decades into the future to prevent AIDS in the millions of HIV-infected people, and to control the spread of the HIV pandemic. But unraveling the relevant mechanisms of proviral latency may allow the development of therapeutic strategies that eradicate infection. Some strategies have already emerged from our current understanding, but because these approaches have thus far been impractical or unsuccessful, new discoveries and further translational effort are needed [32].

Activating Expression of Latent HIV

Several kinase agonists, including hexamethylbisacetamide, a compound previously tested in human cancer trials, activate intracellular signaling cascades that mobilize p-TEFb in the absence of Tat and can induce the expression of HIV in latently infected cells. Prostratin, a nontumorigenic phorbol ester isolated from the Samoan medicinal plant *Homalanthus nutans*, induces HIV expression in latently infected cell lines and cells isolated from HIV-infected, highly active ART-treated patients in the absence of cellular proliferation [7]. Recently, novel phorbol esters and molecules have been described that act like NF-κB but do not induce the cell surface marker phenotype of T-cell activation [33–35]. These promising approaches must overcome the hurdles of potential cellular toxicities and undergo cautious testing to avoid the induction of more virus expression than can be contained by ART [36].

IL-7, a cytokine essential for maintenance of T-cell homeostasis, can induce HIV expression from quiescent resting cells without global T-cell activation, via the JAK/STAT5 signaling pathway. This cytokine has recently been studied for use in HIV-infected patients and thus might be tested for its ability to purge quiescent HIV genomes [37–39]. However, the findings of Chomont et al. [31••] raise the concern that IL-7 exposure could induce the homeostatic proliferation of latently infected resting memory cells.

HDACi as Potential Therapeutics

Because HDAC recruitment to the LTR is required to maintain latency, HDACi should disrupt proviral quiescence. ChIP assays performed in latently infected T-cell lines containing a single integrated HIV genome documented histone H4 acetylation at the nucleosome about the LTR transcriptional start site upon exposure to the HDACi VPA. VPA did not alter de novo HIV infection or the activation phenotype of primary cells, but it induced viral outgrowth from the resting CD4+ cells of patients without activation [21].

These preclinical studies led to a translational experiment, examining the effects of the administration of the HDACi VPA with intensified ART to HIV-infected patients. Initial findings suggest the potential of HDACi as anti-latency therapeutics, and the first targeted approach to this reservoir of persistent HIV infection [40]. A significant decline in resting cell infection (RCI) was measured in three of four patients (mean reduction 70%; range 58% to >84%) after intensification of ART and the addition of VPA. Because reductions observed in three of four patients exceeded the 50% threshold pre-established as being greater than any that would be expected by natural decay of this reservoir, intensified ART, or the variation of our assay, findings suggested that treatment with an HDACi and intensified highly active ART safely depleted RCI in vivo.

Subsequently, the effect on RCI of the addition of VPA to standard ART was tested. There was a significant decline in RCI in four of 11 patients receiving VPA therapy combined with standard ART, and a nonsignificant decline in two others. Patients without a decline in RCI were more likely to have low-level (1–50 copy/mL) viremia [41]. However, no significant change in RCI during periods of VPA treatment for neurologic and psychiatric disorders was reported, using an assay with a larger apparent intra-assay variation [42], and the average frequency of resting cells with detectable HIV RNA was no different in 10 patients taking valproate compared with patients taking only ART [43]. Thus, although VPA provides an important proof-of-concept that HDACi might provide a therapeutic approach to virus eradication, the preliminary clinical evidence available suggests that taken alone, the weak, nonselective HDACi VPA is insufficient to induce the required profound and reproducible depletion of resting CD4 cell infection in most clinical scenarios.

More potent HDACi such as suberoylanilide hydroxamic acid have been shown to be capable of inducing the expression of latent provirus [44, 45], but clinical testing of this approach has thus far not been possible. Combinatorial approaches have been modeled in the laboratory [46], and the field may find it challenging to translate such oncologic approaches in clinical testing within stable HIV-infected populations, in whom the acceptable level of risk is likely to be very low.

Thus, further study of the epigenetic regulators of HIV latency is needed, to allow the development of rational approaches to the problem of latent HIV infection and the identification of more effective and safe inducers of the latent proviral populations.

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- •• Of major importance

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Review

Antiretroviral therapy in macrophages: implication for HIV eradication

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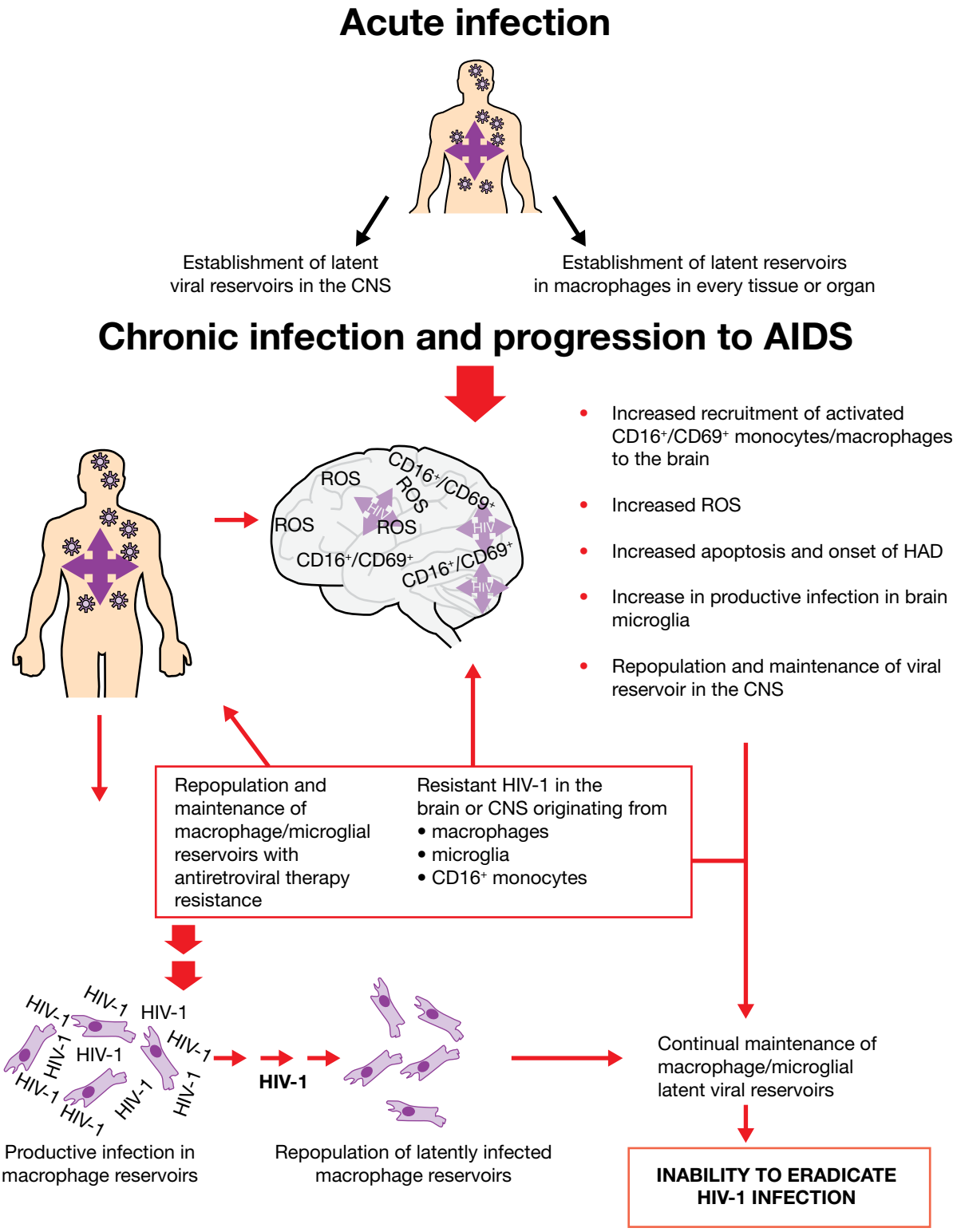
HIV type-1 (HIV-1) accounts for more than 25 million deaths and nearly 40 million people are infected worldwide. A significant obstacle in clearing virus from infected individuals is latently infected viral reservoirs. Latent HIV-1 can emerge with recrudescence as a productive infection later in disease progression and could provide a source for the emergence of resistant HIV-1. It is widely recognized that macrophages represent a latently infected viral reservoir and are a significant and critical HIV-1 target cell *in vivo*. Macrophages can be divided into multiple subsets of macrophage-like cells, all of which are susceptible to HIV-1 infection, including dendritic cells, Langerhans cells, alveolar macrophages, mucosal macrophages and microglial cells. Current antiretroviral therapy (ART) often displays differential antiviral activity in macrophages relative to CD4+ T-lymphocytes. Significant work has been performed to establish antiviral activity of many clinically approved ART in macrophages; however, a direct link between antiviral activity and specific mechanisms responsible for these antiviral effects are incompletely understood. This review identifies many understudied areas of research, along with topics for further research in the field of HIV therapy and eradication. Discussion focuses upon the known cellular pharmacology and antiviral activity of antiretroviral agents in macrophages and its relationship to latency, chronic HIV-1 infection and therapeutic strategies to eradicate systemic HIV-1 infection.

Introduction

Macrophages are a major and crucial target of HIV type-1 (HIV-1) infection and, as potential long-term HIV reservoirs, infected cells must be selectively destroyed to achieve HIV-1 eradication [1]. As macrophages function as potent antigen-presenting cells and mediators of both innate and acquired immunity, HIV-1-mediated macrophage deficiency is catastrophic to the global immune response. A significant obstacle in clearing virus from infected individuals is multiple latently infected viral sanctuaries (Figure 1). Latent HIV-1 can emerge with recrudescence as a productive infection later in disease progression and might also represent a source for the emergence of resistant HIV-1 [2–4]. Many regimens eventually fail, primarily because of lack of adherence to strict regimens, delayed toxicities and/or the emergence of drug-resistant HIV strains [5]. Several studies have begun to elucidate the relationship between antiretroviral therapy (ART) and CD4+ T-lymphocytes relative to toxicity, resistance, latency and chronic infection (reviewed in [6]). Recently, Richman *et al.* [6] presented a comprehensive review commenting on strategies to reduce or eliminate latent HIV-1 infection within the CD4+ T-lymphocyte compartment. This review presented a model wherein latent CD4+ T-lymphocytes could be reduced or eliminated by combination therapy designed to purge remaining latent CD4+ T-lymphocytes in tandem with novel therapeutics targeting pathways implicated as modulators of HIV-1 latency, including histone deacetylase inhibitors and modulators of Jak/STAT-, PKC-, NF-κB- and IL-7-mediated signal transduction cascades [6]. The application of this hypothesis within macrophages is undefined, but presents an integral overlap to facilitate greater understanding of the relationship between macrophages and ART cellular pharmacology.

The function of macrophages *in vivo* presents a long-lived target for HIV-1 infection; the half-life ($t_{1/2}$) of macrophages is significantly longer than that of an activated lymphocyte (weeks/years versus hours/days) [7,8]. More specifically, the life span of activated HIV-1-infected lymphocytes is relatively attenuated, with

Figure 1. Mechanisms for establishment and maintenance of HIV-1 reservoirs in macrophages



Acute HIV type-1 (HIV-1) infection results in establishment of infection in macrophages in the central nervous system (CNS) and every organ or tissue. Chronic infection results in increased recruitment of activated CD16+/CD69+ monocytes/macrophages to the brain, increased reactive oxygen species (ROS) and apoptosis followed by onset of HIV-associated dementia (HAD). These mechanisms increase the productive infection in brain microglia and contribute to maintenance of an HIV-1 viral reservoir in the CNS. Resistant HIV-1 in the brain or CNS along with latently infected macrophages originating from other tissues and organs contributes to maintenance of macrophage viral reservoirs. Together, these factors contribute to the inability to eradicate systemic HIV-1 infection [2,4,7,13,16,24,27,28,31–33,75,77–79,84–90,110].

a $t_{1/2}$ of approximately 0.8–1.1 days [9], whereas productively infected macrophages maintain viability and virus production for at least 30 days [10]. The latter study represents an *in vitro* assay and, although studies observing the $t_{1/2}$ of HIV-1 infected macrophages *in vivo* are lacking, existing studies define a distinct advantage for macrophages when observing their life span and virus production over time. Viral dynamics *in vivo* indicate that CCR5 (R5)-using viruses predominate early during infection [11]. As macrophages display high CCR5 expression levels, they represent an early target for the establishment of both chronic and latent infection [2,4]. Macrophages interact with lymphocytes during antigen presentation, conferring direct infection to new $CD4^+$ T-lymphocytic targets [12]. Macrophages have also been implicated as the causative agent in central nervous system (CNS) infection of HIV-1, which often manifests itself as HIV-associated dementia (HAD) during the later stages in the progression to AIDS [13]. For these reasons, understanding dynamics of ART pharmacology in macrophages, and subsequently eliminating productive infection in these cells, is critical to eliminating systemic HIV-1 infection.

Viral dynamics in macrophages is unique because these cells can be found in every organ system [2,3]. This represents multiple microenvironments from which HIV-1 can establish latent infection and in which ART is often present with significantly different antiviral activity profiles relative to circulating $CD4^+$ T-lymphocytes [14,15]. Studies are conflicting as to the proposed mechanism(s) responsible for observed antiviral activity of ART in macrophages. Suggested mechanisms differ relative to the class of ART, activation state of the target cell, drug concentration and time after initial infection that cells are exposed to drug [16–18].

The efficacy of ART cellular pharmacology in macrophages has significant implications in disease progression. The interplay between ART cellular pharmacology in macrophages directly affects viral loads, selection of resistance mutations both within and between subsets of HIV-1 target cells, eradication of systemic virus and long-term patient survival [2,10,13,16,19–30] (Figure 1). Eradication of systemic HIV-1 infection is not possible without clearance of latently infected cells [4,13,29–36]. As macrophages are a sentinel target for HIV-1 infection and latency, understanding the cellular pharmacology of current antiretroviral therapy in macrophages is essential.

This manuscript provides state-of-the-art knowledge on HIV-1 replication in macrophages, especially relating to antiretroviral agents, and also defines understudied areas of research. It should be noted that the general term 'macrophage' is used here to indicate cells that are obtained from the circulating peripheral blood. However, *in vivo*, macrophages can be broadly categorized by mechanism of activation into classically activated (M1) and alternatively activated (M2) macrophages [37–40]. M1 classification is associated with prototypical secretion of proinflammatory cytokines interleukin (IL)-1β, IL-15, IL-18, IL-12 and tumour necrosis factor (TNF)-α, which modulate enhanced endocytic function coupled with increased capacity to eliminate intracellular pathogens [37–40]. M2 macrophages can be further classified into M2a, M2b, and M2c subsets, each with distinct functionalities and cytokine profiles [37–43]. In this review, the potency and efficacy of ART for each subset of macrophages are not defined nor are the dynamics of HIV-1 infection within M1 or M2 macrophages.

Entry inhibitors

Currently, only two anti-HIV drugs are approved by the US Federal Drug Administration (FDA) that inhibit entry of HIV-1 into host cells (Table 1). Enfuvirtide (Fuzeon®; ENF, T-20) is a peptide derived from a repeat sequence of the transmembrane portion of HIV-1 envelope, gp41, and inhibits the hairpin formation necessary for virus–host cell fusion to occur [44]. Maraviroc (Selzentry®/Celsentri®; UK-427, 857) is a small molecule that inhibits viral entry by binding to the CCR5 coreceptor and inhibiting the receptor–coreceptor viral envelope interaction required for HIV-1 entry into the host cell [45,46].

Maraviroc has undergone extensive plasma pharmacokinetics and testing against multiple HIV-1 variants and clades [46]; however, studies assessing antiviral activity, toxicity or cellular pharmacology in macrophages are largely undefined. The median effective concentration (EC_{50}) for maraviroc in primary macrophages is reported to be 0.5 nM for a subtype B M-R5 virus [29] versus 0.2–2.9 nM in activated lymphocytes for R5-using viruses across multiple subtypes [46] (Table 1). Follow-up studies across multiple donors and within macrophages at different stages of activation are not fully elucidated; therefore, one should always compare potency of antiviral agents in the same assay using the same experimental procedures.

Extensive studies assessing antiviral activity of either maraviroc or ENF in primary macrophages are lacking, although Yi *et al.* [47] recently offered data comparing the sensitivity of ENF upon CCR5- versus CXCR4-mediated HIV-1 entry into primary macrophages. ENF sensitivity was largely independent of the coreceptor usage, and overall ENF antiviral activity in macrophages correlated more closely with HIV-1 entry by CCR5 rather than with entry through CXCR4 [47] (Table 1). These data demonstrate the downstream effects of ENF relative to CXCR4- versus CCR5-mediated signalling and provide

Table 1. Potency of currently approved antiretroviral therapy in macrophages versus PBMCs

Compound	Acute infection in macrophages EC_{50}, nM	Chronic infection in macrophages EC_{50}, nM	Acute infection in PBMCs EC_{50}, nM
Entry inhibitors			
Maraviroc	0.5	NA	0.2–2.9
Enfuvirtide	20	NA	NA
NNRTI			
Delavirdine	10	NE	6
Efavirenz	10	NE	10
Etravirine	NA	NA	NA
Nevirapine	50	NE.	40
NRTI			
Abacavir sulfate	300	NE.	NA
Didanosine	50	NE	500
Lamivudine	20	NE	40
Stavudine	240	NE	800
Tenofovir disoproxil fumarate	20	NE	370
Zalcitabine	3	NE	40
Zidovudine	20	NE	200
Protease inhibitors			
Amprenavir	10	720	NA
Atazanavir	NA	NA	NA
Darunavir	NA	NA	NA
Fosamprenavir	NA	NA	NA
Indinavir	60	400	50
Nelfinavir	80	1.4×10^3	40
Ritonavir	120	3.3×10^3	20
Saquinavir	50	500	10
Tipranavir	NA	NA	NA

The 50% effective concentration (EC_{50}) of US Federal Drug Administration-approved HIV type-1 antiretroviral therapy in acute versus chronically infected macrophages [14–17,22,29,35,45–47,52,119]. NA, not available; NE, not effective; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PBMCs, peripheral blood mononuclear cells.

a foundation for understanding the effect of entry inhibitors on multiple cellular events in macrophages.

Reverse transcriptase inhibitors

Two classes of reverse transcriptase (RT) inhibitors exist for treatment of HIV-1 infection: nucleoside RT inhibitors (NRTIs; nucleoside analogues) and non-nucleoside RT inhibitors (NNRTIs). NRTIs have a well established regulatory pathway, with eight FDA-approved drugs for the treatment of HIV-1 infection and multiple drugs in various stages of clinical development [48,49]. NNRTIs also have a long history of FDA approval, with four drugs currently approved.

Nucleoside reverse transcriptase inhibitors

NRTIs present distinct clinical advantages: low plasma protein binding, sustained antiviral response when a dose is missed (because of their long intracellular half-life) and relative ease of chemical manufacture [48]. By 2008, the eight approved HIV-1 NRTI were zidovudine (Retrovir®; ZDV, AZT), didanosine (Videx®; ddI), zalcitabine (Hivid®; ddC), stavudine (Zerit®; d4T), abacavir sulfate (Ziagen®; ABC), lamivudine (Epivir®; 3TC), emtricitabine (Emtriva®; FTC) and tenofovir disoproxil fumarate (Viread®; TDF).

The target of NRTIs in HIV-1 infection is the action of virally encoded RT. This enzyme is active early in the viral replication cycle and converts the genetic information of the virus, which is stored as RNA, into DNA by reverse transcription, a process necessary for continued viral replication [48,49].

NRTIs are chiral small molecules that mimic natural nucleotides and require intracellular phosphorylation to become functionally active against HIV-1 RT. In the triphosphate form, NRTIs compete with one of the four naturally occurring dNTP, namely, dCTP, dTTP, dATP or dGTP, for binding and DNA chain elongation near the active site of HIV-1 RT [48]. As most NRTIs lack a 3′-hydroxyl terminus, incorporation of the analogue into the growing DNA strand results in termination of the DNA strand and the next phosphodiester bond is not formed. Because of these factors, both the concentration of cellular triphosphorylated drug and the levels of cellular dNTP pools play a key role in the efficacy of the NRTIs [49,50].

Antiviral activity of nucleoside reverse transcriptase inhibitors in macrophages

The antiviral activity of NRTIs in macrophages is significantly different for acute versus chronically infected cells. Mechanisms responsible for these observations are currently undefined, although multiple hypotheses exist to explain these findings. Natural dNTP levels have been historically demonstrated as a surrogate marker for replication and activation of mammalian cells [51]. Macrophages primarily remain in a resting, G1 state and undergo limited DNA synthesis [7,51,52]. Therefore, it follows that cellular dNTP levels are significantly lower in macrophages compared with an activated and dividing cell [15]. For acute infection, NRTI EC_{50} in macrophages demonstrates ranges from 3 to 300 nM depending on the NRTI tested (Table 1). By contrast, data for chronically infected macrophages demonstrate an EC_{50}>25 μM or state that NRTIs are 'not effective' in chronically infected macrophages [16]. These differences indicate that chronic HIV-1 infection alters the cellular milieu in a manner that modulates the ability of NRTI to successfully become functionally active and/or subsequently inhibit viral reverse transcription. The significant difference in EC_{50} might be explained in part by differences in activation state, which could, in turn, affect dNTP levels. As NRTIs compete with endogenous nucleotides for incorporation into the growing viral DNA, differences in dNTP levels, as a function of activation state, could significantly alter NRTI-triphosphate levels.

Alternatively, the difference in number of infected cells in an acute versus chronic infection *in vitro* might affect the potency of the NRTIs. A chronically infected culture possesses a greater number of infected cells, which, in turn, can induce a cytokine milieu that promotes infection [53,54]. This milieu might modulate a globally activated system, which either reduces intracellular bioavailability of NRTIs or increases its catabolism. Interestingly, Aquaro *et al*. [14] reported that the constant exposure of macrophages to monocyte colony-stimulating factor (m-CSF) results in a significant increase in EC_{50} (Table 1), which might provide evidence of activation state affecting potency of NRTI.

As chronically infected cells contain an integrated proviral genome, which is downstream of the NRTI target, it follows that NRTIs will not be as effective in a population of cells containing integrated HIV-1. Despite this fact, a chronic infection contains a mixture of cells at various stages of infection. Newly established infection continues to occur, thus providing a functional target for NRTIs in some cells. Although integrated proviral genome is a characteristic of chronic infection, it is important to note that not all cells *in vitro* or *in vivo* are infected even as chronic infection is established. Thus, the mechanism of action of NRTIs along with its inability to inhibit viral replication in cells with integrated HIV-1 DNA is not applicable to all cells and to new infection established in cells neighbouring chronically infected cells. Whether the activation state (and thus the endogenous cellular dNTP pools) in neighbouring cells is altered as a function of paracrine stimulation from chronically infected neighbour cells is not defined. Together, the knowledge to date suggests a multimechanism system wherein NRTIs might not be as effective in chronically infected cells because of multiple factors.

The EC_{50} of NRTIs for acute infection in macrophages relative to $CD4^+$ T-lymphocytes is significantly lower. Although the mechanisms are unknown, it follows that differences between cell types might be responsible for these data. These findings have led to the hypothesis that lower cellular dNTP pools in resting macrophages (compared with that of lymphocytes) results in decreased competition for cellular kinases. Thus, the ability of NRTI to undergo phosphorylation is increased, resulting in greater antiviral activity. A tandem hypothesis is that during chronic HIV-1 infection continuous, exposure to m-CSF might activate the macrophage resulting in increased dNTP levels. This, in turn, would increase competition for cellular kinases and decrease the ability of the NRTIs to become functionally active. Together, these mechanisms might be responsible for markedly different EC_{50} values for analogous drugs across macrophages and lymphocytes.

Viral resistance to nucleoside reverse transcriptase inhibitors in macrophages

Macrophages represent a long-lived reservoir for HIV-1 infection and survive for weeks or years within an infected individual [3,7,22]. Understanding the dynamics of ongoing or latent infection during the emergence of resistant HIV-1 is essential for managing chronic infection [55], drug regimens and the design of novel therapeutics that might confer less resistance for extended periods of time.

Studies assessing the effect of 3TC on the emergence of drug-resistant HIV-1 in macrophages over time surprisingly demonstrated a lack of emergence of resistance mutations [23]. When 3TC treated acutely infected macrophages exposed to supernatants that were transferred from infected macrophages (assay conducted for 105 days), no resistance mutations were observed, despite emergence of mutations that confer resistance to NRTI in lymphocytes [23]. These data suggest that the dynamics of 3TC in lymphocytes versus macrophages is different, and that the mechanisms responsible for the emergence of resistance in lymphocytes are not uniform in macrophages. The relationship between fitness and cell-specific emergence of resistant mutations was not defined, although potential differences

in replicative kinetics across cell types might contribute to these findings. Other experiments demonstrate a resistance-associated loss of replicative capacity in acutely infected macrophages relative to activated CD4+ T-lymphocytes [56]. These data define the relationship between acute infection, NRTI activity and emergence of resistance. They also suggest a role for resistance-associated loss of fitness in macrophages.

Although it is favourable to correlate the lack of resistance mutations over time with NRTI treatment, the effect of NRTI on chronically infected macrophages is difficult to elucidate. Chronic infection directly relates to latency because latent infection often emerges later in disease progression. Although specific mechanisms responsible for reactivation *in vivo* are not fully understood, it is accepted that reactivation of previously latently infected macrophages can result in repopulation of the systemic system with virus, which contributes to disease progression (reviewed in [1,2,6,57]). It is well established that NRTI EC_{50} in activated and/or chronically infected macrophages is very high or cannot be established *in vitro* (Table 1), which provides a difficult environment from which to assess studies more easily defined in resting macrophages or CD4+ T-lymphocytes.

Non-nucleoside reverse trancriptase inhibitors

By 2009, four NNRTIs were approved by the FDA: etravirine (Intelence®; TMC-125), delavirdine (Rescriptor®, DLV), efavirenz (Sustiva®, Stocrin; EFV) and nevirapine (Viramune®; NVP). NNRTIs are chemically distinct from NRTI and inhibit HIV-1 RT by a different mechanism. NNRTIs interact with binding pockets of HIV-1 RT, inhibiting its enzymatic activity by causing conformational changes at or near the active site [58].

Antiviral activity of NNRTI in macrophages

Although the cellular dNTP pool does not directly affect the mechanism of action of NNRTIs, the EC_{50} differs significantly for NNRTI against acute versus chronic infection in macrophages (reviewed in Aquaro *et al.* [16]). The EC_{50} for inhibition of acute HIV-1 infection in macrophages ranges from 10 to 50 nM depending on the NNRTI; however, NNRTIs are not effective at inhibiting HIV-1 replication in chronically infected macrophages (Table 1). To date, the mechanism(s) responsible for ineffective inhibition of viral replication in macrophages by NNRTI (and NRTI) are incompletely understood. Similar principles as discussed with NRTI might be partially responsible for these data (establishment of integrated proviral genome downstream of NNRTI target). If this mechanism was solely responsible for differential activity of NNRTI in chronically activated macrophages, significant antiviral activity would probably still be observed because establishment of new infection and subsequent p24 production would be terminated. As observed in human lymphocytes, NRTI and NNRTI do not efficiently inhibit HIV-1 infection in chronically infected macrophages and data defining the mechanisms are lacking. NRTIs and NNRTIs cannot affect virus replication once the virus is integrated into the host genome. Additional studies elucidating the relationship between NRTIs, NNRTIs and HIV-1 infection in macrophages, could address current gaps in knowledge and allow for design of drugs with greater potency in populations of chronically infected macrophages, such as protease inhibitors (PIs), which are known to be effective in chronically infected cells (see below). Although many data correlate a significantly higher EC_{50} for activated macrophages versus lymphocytes, data observing intracellular accumulation of NNRTI are lacking. Intracellular accumulation of NNRTI within macrophages might present with a significantly diminished intracellular bioavailability profile relative to lymphocytes, which could present a direct link between differential activities of NNRTI in macrophages versus lymphocytes. Further experiments should be conducted to define the links between latent infection in macrophages, chronic HIV-1 infection and antiviral activity of both NRTIs and NNRTIs in macrophages at all stages of activation and infection.

Integrase inhibitors

HIV-1 integrase (IN) presents a novel and highly selective target for anti-HIV therapeutics. Raltegravir (Isentress®; MK-0518, RAL) received FDA approval in October 2007 and acts to inhibit integration of HIV-1 proviral genome into host cell DNA [59]. To date, studies are lacking in human macrophages. Future studies with RAL in macrophages might define any long-term relationship between IN inhibitors and toxicity, therapy outcome, chronic infection, establishment and emergence of resistant mutations, and latency.

Protease inhibitors

HIV-1 protease inhibitors (PIs) represent a class of ART with long-standing FDA approval. To date, 10 PIs have received FDA approval: amprenavir (Agenerase®; APV), tipranavir (Aptivus®; TPV), indinavir (Crixivan®; IDV), saquinavir (Invirase®; SQV), lopinavir/ritonavir (Kaletra®, Aluvia; LPV/r), fosamprenavir (Lexiva®, Telzir; FPV), ritonavir (Norvir®; RTV), darunavir (Prezista®; DRV), atazanavir (Reyataz®; ATV) and nelfinavir (Viracept®; NFV). PR cleaves HIV-1 Gag and Gag–Pol, resulting in a mature, infectious virion. PIs compete for binding in the active site with the natural

substrate. Once bound, PIs cannot usually be cleaved, resulting in inactivation of the enzyme [60].

The hallmark of a PI is its ability to inhibit HIV-1 replication in macrophages (and lymphocytes) chronically infected with HIV-1. The EC_{50} of multiple PIs have been observed against both acute and chronic HIV-1 infection in macrophages (reviewed in [15,16]; Table 1). Unlike NRTIs and NNRTIs, PIs display potent activity in both acutely and chronically infected macrophages. Of the PIs tested, the EC_{50} in acutely infected macrophages ranged from 10 to 120 nM, whereas the EC_{50} in chronically infected macrophages ranged from 400 to 3.3×10^3 nM (Table 1). Some reports correlate higher rates of viral RNA metabolism in macrophages (relative to $CD4^+$ T-lymphocytes) as a mechanism responsible for higher EC_{50} in macrophages [61]. As viral RNA production is mechanistically distinct from the action of PIs, it follows that this might contribute to higher EC_{50} of PIs in macrophages.

Understanding potential mechanisms responsible for antiviral activity of currently available ART is crucial. Correlating *in vitro* data with *in vivo* viral–host cell dynamics presents a unique and challenging obstacle. Plasma pharmacokinetics *in vivo* demonstrate inhibitory quotient values (minimum concentration [C_{min}]/EC_{50} value, which adjusts for plasma protein binding) that are similar to the EC_{50} of PI *in vitro* for inhibition of chronic HIV-1 infection in macrophages [62]. Macrophages are unique because they can be found in every tissue compartment and organ systemically. Therefore, it is reasonable to suggest that many macrophages *in vivo* might be exposed to significantly lower levels of drug than those observed at inhibitory quotients or C_{min} in plasma in the circulating periphery.

Data observed for chronically infected macrophages *in vitro* must be carefully compared with known pharmacology of drugs *in vivo*. As is the case with PIs, it is clear that antiviral activity is demonstrated, which might correlate with specific subsets of macrophages either in the circulating periphery or in specific tissue compartments displaying high bioavailability of drug. Levels of PI below the inhibitory quotient or C_{min} in macrophages might represent a viral reservoir for the emergence of resistant viruses. Understanding specific interactions and mechanisms responsible for antiviral activity of PIs in macrophages and the relationship between differential activity of PI in macrophages at different levels of activation or exposure to HIV-1 infection is essential in abolishing establishment of latent HIV-1 infection. This area of study represents a rich foundation for continued studies to understand how antiviral activity and pharmacology of ART in macrophages contribute to establishment and maintenance of latent infection.

Toxicity of ART in macrophages

It is well established that administration of any ART is not always without detrimental consequence. Studies have extensively demonstrated a link between inhibition of mitochondrial DNA (mtDNA) polymerase-γ, mitochondrial toxicity and systemic side effects including cardiomyopathy [63]. PI use has been correlated with inhibition of apoptosis via multiple mechanisms in lymphocytes [64]; however, the effect of PI in macrophages is largely undefined. The toxicology profile of ART in macrophages remains a greatly understudied area of research.

Azzam *et al.* [65] demonstrated that treatment of macrophages with either ddI/d4T or 3TC/AZT results in a decrease in mtDNA content in both HIV-1-infected and -uninfected macrophages. The effect was more pronounced in infected macrophages. These observations were correlated with a decrease in complement-mediated phagocytosis, suggesting impairment in immune function. These findings were only observed with coadministration of multiple NRTI, demonstrating a potential synergistic toxicological effect. As the concentrations at which mtDNA content was impaired were ≥10 μM, this presents a debate as to the physiological relevance of these data relative to *in vivo* pharmacokinetics of NRTI. Nonetheless, these data demonstrate a system where, in concert, NRTI might result in mtDNA depletion in macrophages.

Relationship between ART and dendritic cells

Dendritic cells are macrophage-like cells that can be divided into two distinct subpopulations termed myeloid dendritic cells and plasmacytoid dendritic cells, both of which are susceptible to HIV-1 infection and express varying levels of both CD4 and chemokine coreceptors CXCR4 or CCR5 [66]. Dendritic cells are considered an early target for establishment and maintenance of infection, and represent a distinct viral reservoir [24]. These cells are potent antigen-presenting cells and have been implicated not only as a viral reservoir, but also as a mechanism of viral transmission to $CD4^+$ T-lymphocytes during antigen presentation [24,66]. Dendritic cells are migratory, representing a mobile viral reservoir capable of infecting lymphocytes, as well as other macrophage-like cells, across multiple tissue compartments.

These cells are also primary modulators of both innate and acquired immunity, primarily by immune responses mediated by IL-12 and interferon-α, and stimulate clonal expansion of naive T-lymphocytes [24,66,67]. Chronic HIV-1 infection depletes levels of circulating dendritic cells, which directly correlates with increases in viral loads, decrease in $CD4^+$ T-lymphocytes counts,

significant immunological impairment hallmarked by opportunistic infection and progression to AIDS [24,66]. Dendritic cells represent a subpopulation of macrophages that are important modulators of functional immunity, while simultaneously acting as a viral reservoir and mechanism of transfer of HIV-1. Despite the sentinel role of dendritic cells in HIV-1 infection, and the obstacle that they represent in eradication, the pharmacological profile of FDA-approved ART in dendritic cells remains largely undefined.

Although EC_{50} values for many FDA-approved ART are defined for acute and chronic HIV-1 infection in macrophages [3,14–16,22,29,52], these data are lacking within dendritic cells. It follows that the cellular pharmacology of all FDA-approved ART are undefined within dendritic cells. Despite a lack of studies observing antiviral activity and cellular pharmacology of current ART, emerging studies have begun to exploit the antigen-presenting capacity of dendritic cells with therapies designed to modulate the immune response to HIV-1 [67–70].

DermaVir is a novel therapeutic in development by Genetic Immunity [70], which contains non-infectious viral DNA packaged with a 100 nm nanoparticle. The complex is designed to mimic the antigenic peptides present on dendritic cells, conferring enhanced immunity upon exposure of $CD4^+$ T-lymphocytes to the antigenic peptides. Activation of HIV-1-specific lymphocytes from a previously naive pool would, in principle, expand effector and memory cell populations, resulting in a decrease in viraemia. Long-term data correlating the efficacy and safety of this therapeutic approach and its ability to affect dendritic cells across multiple tissue compartments and in distinct localizations, remains an ongoing area of research.

Relationship between ART and alveolar macrophages

Alveolar macrophages are localized to the pulmonary alveolus and primarily provide innate immunity by phagocytosis of extracellular micro-organisms and pathogens encountered during respiration. Alveolar macrophages express CD4, CXCR4 and CCR5, and are permissive to both CXCR4- and CCR5-using HIV-1 [71]. Although alveolar macrophages might represent a subset of cells functioning as viral reservoir for persistence and maintenance of HIV-1 [71,72], and are therefore a potential obstacle in the eradication of systemic HIV-1, studies elucidating the antiviral activity and cellular pharmacology of ART are lacking. Early work established that maintenance of AZT in alveolar macrophage cultures inhibits productive transfer of infection to lymphocytes [73,74]. However, further work with other ART, either alone or in combination, and complementing pharmacological data, are undefined.

The role of cytokines in modulating HIV-1 infection in alveolar macrophages is partially understood, as TNF-α, a proinflammatory cytokine associated with progression to AIDS [53,54,75,76], induces down-regulation of CCR5 surface expression coupled with paracrine and autocrine stimulation of MIP1-α, MIP1-β and RANTES, which are natural ligands for CCR5 [76]. Together, these cytokines in combination with decreased CCR5 expression might serve as complementing cytoprotective mechanisms in the presence of CCR5-using HIV-1 [76]. Together, these data establish a fundamental relationship between cytokines, CCR5 expression and HIV-1 pathogenesis, and provide an excellent foundation for studies elucidating the effect of FDA-approved CCR5 inhibitors or novel immune-based therapies on HIV-1 replication in alveolar macrophages. Novel strategies implementing small molecules designed to modulate cytokine expression, in particular TNF-α, within alveolar macrophages could provide a unique foundation for reduction and elimination of virus within distinct tissue reservoirs.

Relationship between mucosal macrophages and HIV-1 infection

Mucosal surfaces are densely populated with both macrophages and lymphocytes (reviewed in [77]). As HIV-1 infection is acquired in most cases through direct interaction with mucosal surfaces [77], understanding the dynamics for establishment of productive and latent infection, and its relationship to potency of ART within mucosal macrophages, represent critical questions that must be addressed before eradication can occur. Although studies defining the potency of ART across subsets of mucosal macrophages are lacking, a recent study by Shen *et al.* [77] provided insights on HIV-1 permissiveness of multiple subsets of mucosal macrophages.

These data define the receptor expression profile of vaginal and intestinal macrophages, and correlate surface expression of CD4, CXCR4, CCR5, CD14, CD89, CD16, CD32 and CD64 in vaginal mucosal macrophages with permissiveness to an R5-using HIV-1. In contrast, intestinal macrophages displayed low or absent receptor expression of each of the reported innate immune response receptors expressed in vaginal macrophages, and low or absent HIV-1 receptor and coreceptor expression. These data correlated with the inability to observe productive HIV-1 infection within intestinal macrophages. Together, these findings demonstrate the role of vaginal macrophages in establishment of acute infection and implicate a potential role for vaginal macrophages as a viral reservoir. Although

the potency of ART across vaginal mucosal macrophages is currently unknown, defining the potency of current and novel ART within a subset of cells representing a front-line innate immune response to HIV-1 infection could serve as a valuable tool for design of novel therapeutics specifically targeting these cells. The data reported in Shen *et al.* [77] provide a foundation for extensive studies designed to elucidate the antiviral activity of ART against CCR5, CXCR4 or dual-tropic HIV-1 in vaginal macrophages.

Relationship between CD14lo/CD16hi monocytes and HIV-1 infection

A unique subset of non-terminally differentiated macrophages is the CD14lo/CD16hi monocytes. This subset of monocytes has been demonstrated to represent the approximately 10–15% of monocytes that are permissive to HIV-1 infection [78,79] and possess many macrophage and dendritic cell-like properties including high CCR5 and MHCII expression, and the ability to act as a migratory surveyor of multiple tissues [78,79]. As both tissue macrophages and dendritic cells perform similar functions, it follows that CD14lo/CD16hi monocytes might represent a significantly understudied viral reservoir (Figure 1). To this end, Jaworoski *et al.* [79] recently reported that the predominant site of HIV-1 infection within monocytes *in vivo* is the CD16hi subset. Although antiviral activity and cellular pharmacology of ART in CD14lo/CD16hi monocytes represent an unstudied area of research, novel drug design targeting monocyte subsets based on receptor expression profiles might present an area for future drug design.

Relationship between ART, microglial cells and the central nervous system

Microglial cells represent a significant target for HIV-1 infection within the CNS [25,32,80,81] and predominantly express CD4 and CCR5; however, subsets of microglial cells express CXCR4 and are permissive to CXCR4-using or dual-tropic (R5X4) viruses [26,82]. The CNS provides a distinct microenvironment because infection is established during acute infection, but remains largely latent until later in disease progression [83,84] (Figure 1). Factors including diminished immune function and $CD4^+$ T-lymphocyte counts coupled with increased plasma viral loads, contribute to a systemic increase in infected, activated $CD14^+/CD16^+$- and $CD14^+/CD69^+$-expressing monocytes [85–87]. This, in turn, triggers increased trafficking of infected cells across the blood–brain barrier (BBB), which, in combination with the activation state of the cells, stimulates reactivation of latent infection within microglial cells in the CNS [86,87].

Clearance of both latent and productive infection in the CNS is essential in eradication of HIV-1 [32] and presents an immediate concern for current patients because HAD occurs in approximately 20% of HIV-infected individuals and is responsible for significant neurocognitive impairment [28]. Disease progression and increased viral loads in the CNS and brain are also associated with increased levels of activated monocytes and microglial cells [85,88], which correlate with progression to AIDS and an increased occurrence of either minor cognitive motor disorder (MCMD) or the more severe HAD [89,90]. Although the distinct mechanisms responsible for MCMD and HAD in HIV-infected persons are not fully elucidated, a link between activated monocytes, microglial cells and macrophages in the brain, and manifestation of clinical symptoms has been drawn [85,88–90]. Increased levels of activated cells of the monocytic lineage in the brain results in increased production of proinflammatory cytokines TNF-α, IL-1 and interferon-α [88–90], which function in a paracrine fashion to confer neuronal death and ultimately results in MCMD or HAD. Studies are conflicting when determining the relationship between administration of ART and the ability to eliminate or reduce MCMD and HAD [91,92], although Carvalhal *et al.* [93] correlates ZDV 3TC plus EFV treatment with decreased cognitive motor impairment and HAD. These data correlated with a statistically significant decrease in CNS viral loads, demonstrating that the effect of ART on MCMD and HAD might be an indirect mechanism facilitated through a decrease in overall viraemia. Although the interplay between ART and macrophages in MCMD and HAD is not fully elucidated, it is plausible that reduction of viraemia in the brain/CNS by some ART could correlate with decreased levels of activated monocytes/macrophages; therefore, decreasing cytokine-mediated neuronal death [85,87,89,90,94] (Figure 1). These data demonstrate the correlation between ART administration and decreased cognitive motor impairments, and highlight the need for novel therapeutics with greater BBB penetration.

Penetration of current ART across the BBB and in the CNS is poor, conferring a microenvironment wherein suboptimal levels of drug allow for emergence of mutations that confer resistance to ART, and the establishment and maintenance of viral reservoirs [57,95,96]. Understanding the cellular pharmacology of current ART in the CNS, and within subsets of primary HIV-1 targets within the CNS, remains a significant obstacle in the treatment of HIV-1-infected patients, and the design of novel therapeutics with increased CNS penetration and bioavailability is essential. Fortunately, new animal models for HIV-1 encephalitis in mice have been developed, which will facilitate the development of improved drug targeting within the brain and CNS [97,98].

As current ART display low penetration in the CNS, a focus on alternative mechanisms for reduction and elimination of HIV-1 and HAD in microglial cells is essential. Agrawal *et al*. [99] hypothesized that HIV-1 gp120-induced secretion of cytotoxic reactive oxygen species (ROS) could be reduced or eliminated and therefore examined the effect of vector-mediated delivery of antioxidant enzymes Cu/Zn-superoxide dismutase (SOD1) and/or glutathione peroxidase (GPx1) to primary neuronal cultures. A significant reduction in gp-120-induced apoptosis was observed, demonstrating the importance of non-traditional targets as mediators of HIV-1 infection in the CNS. Whether a vector-mediated delivery of cytoprotective agents can provide sustenance in HIV-1-infected persons, and whether this delivery system provides practical application, are ongoing areas of research. Nonetheless, this study highlights the necessity for reduction in both total viral loads in the CNS and elimination in HIV-1-mediated apoptosis and cytotoxicity. Novel targets with greater CNS penetration and potency represent an understudied area of research and remain a significant obstacle in systemic eradication of HIV-1.

Metabolism of antiretroviral therapy in macrophages

Studies on cellular drug interactions with antiretroviral agents prior to clinical trials are necessary to detect possible drug interactions. Dynamics of additive or synergistic drug effects *in vitro* provides a foundation for understanding how drugs might interact *in vivo*. Multiple studies have observed the effect of coadministration of ART *in vitro* in cell lines and primary heterogeneous lymphocyte populations [100–102]. Few studies have observed dynamics of drug interactions in macrophages. Pilot studies using nanoparticle-indinavir (NP-IDV) provide fundamental data about potential metabolism of NP-IDV in macrophages. Dou *et al*. [103] subjected primary macrophages to a single 50 μM dose of NP-IDV and demonstrated limited cytotoxicity for 6 days, and correlated these data with constant intracellular drug levels and minimal metabolism. As NP-IDV has not been tested *in vivo*, its systemic pharmacokinetic profile was not defined, presenting difficulty in assessing how these data might affect HIV-1 infection *in vivo*. Independent of current gaps in knowledge, this study presents a backbone for understanding metabolism of novel therapeutics in macrophages.

Novel therapeutics and potential targets in macrophages

Discovering novel targets to inhibit HIV-1 replication presents an important area of study. Combination ART remains a backbone of current treatment approaches [20] and the elimination of virus cannot occur by a single mechanism. Many new targets are currently being explored, including those targeting HIV-1 maturation, inhibition of HIV-1 accessory proteins, inhibition of cellular factors involved in viral replication or immune-based treatments [45,47,104–106] (Table 2). To date, most studies focus upon inhibition of viral replication in $CD4^+$ T-lymphocytes, although recent studies have begun to define antiviral activity of novel therapeutics in macrophages. The potential novel therapeutics described in this review focus upon macrophage-related drug targets.

Table 2. Potency of novel antiretroviral therapies in acute versus chronic macrophages

Potential antiretroviral therapy	Acute infection in macrophages EC_{50}, nM	Chronic infection in macrophages EC_{50}, nM
BIT225	1,100	U
Carbohydrate-binding agents	80	U
PI3K/Akt Inhibitors	200[a]	U
siRNAs	NA	NA

The 50% effective concentration (EC_{50}) of HIV type-1 antiretroviral therapy in various stages of development for acute chronically infected macrophages. [a]EC_{50} observed in the presence of inhibitors of nitric oxide. NA, not applicable; siRNA, small interfering RNA; U, undefined.

Small interfering RNAs (siRNAs) could be useful for the induction of potent gene silencing by degradation of cognate RNA. The use of siRNA for HIV-1 infection presents a unique challenge because systemic or directed silencing of CXCR4 coreceptor would result in mortality, and silencing of CCR5 coreceptor could represent a selective pressure for emergence of highly pathogenic CXCR4-using viruses. Independent of HIV-1 infection, sustained silencing in dividing cells is maintained for attenuated time frames (3–7 days); however, sustained silencing in non-dividing, terminally differentiated macrophages was examined *in vitro* as a model with potential therapeutic implications [107]. Replication kinetics of acute HIV-1 infection in primary macrophages stably transfected with CCR5-targeted siRNA alone or in combination with siRNA targeted against HIV-1 structural protein p24 were examined. HIV-1 infection was effectively eliminated when observed over 15 days in macrophages coexpressing CCR5 and p24 siRNAs [107]. Although these results provide a promising foundation, significant complexities of HIV-1 infection in macrophages persist and cannot be rectified with siRNA-mediated inhibition of HIV-1. Toxicity of siRNA in the context of HIV-1 infection is largely undefined, although Anderson and Akkina [108] provided a study presenting various methods of siRNA-mediated knockdown

of CCR5 and their corresponding toxicities in transgenic macrophages. Whether *in vivo* introduction of siRNA would result in significant toxicity is undefined and, in combination with selective pressure for emergence of pathogenic CXCR4-using viruses, continues to present a significant obstacle in the use of siRNA as a therapeutic tool for treatment of HIV-1 infection.

Carbohydrate-binding agents (CBAs) have been described as potential inhibitors of HIV-1 infection [105]. CBAs target the heavily glycosylated HIV-1 envelope, impairing the ability of macrophages or dendritic cells to recognize and perform antigen presentation to $CD4^+$ T-lymphocytes, subsequently impairing transfer infection [12,105]. Pilot studies in acutely infected primary macrophages demonstrate that CBA molecules demonstrate antiviral activity at concentrations as low as 80 nM (Table 2). The toxicological profile or the ability of CBAs to inhibit chronic HIV-1 infection is not currently defined. As the mechanism of action of CBAs is predominantly extracellular, and could effectively inhibit transfer of infection, these drugs might prove efficacious when coadministered with other classes of ART.

PI3K/Akt pathway inhibitors present a novel anti-HIV-1 target that has undergone recent study. The PI3K/Akt pathway is a cell survival pathway that is activated upon apoptotic stress and functions to activate downstream modulators of cell survival [109]. Recent reports have hypothesized that inhibition of the PI3K/Akt pathway might result in super-sensitivity and ultimately cell death in HIV-1-infected macrophages because they are exposed to normal cellular stressors [104]. Chugh *et al.* [104] demonstrated that PI3K/Akt inhibitors could inhibit HIV-1 replication in acutely infected primary macrophages. Interestingly, antiviral activity was only observed when drugs were coadministered with a compound that positively modulates nitric oxide-induced cytotoxicity in HIV-1 infection [110,111]. In addition, concentration of PI3K/Akt inhibitors conferring inhibition of viral replication was 200 nM (Table 2). The effect of these inhibitors on chronically infected macrophages, or macrophages at different stages of activation, is not known. This study provided an interesting novel drug target and demonstrated efficacy at high concentrations. Although this concentration appears high, and unlikely to be physiologically relevant, these data provide a foundation for studies that exploit cellular signalling pathways that are activated in HIV-1 infected, but not uninfected cells.

Vehicle-based delivery of currently approved ART to macrophages remains an ongoing area of research. Peptide fragments designed to bind specifically to macrophages, thus conferring phagocytosis, provide an attractive mechanism to target macrophages and macrophage-like cells. Dutta and Jain [69] recently evaluated the ability of EFV-loaded tuftsin to inhibit HIV-1 replication in primary macrophages. A significant reduction in supernatant p24 levels were observed relative to controls; however, studies defining the relationship between this drug and replicative kinetics for CXCR4, CCR5 or dual-tropic viruses is not fully elucidated. Whether efavirenz-loaded peptides provide a realistic approach for treating infection in humans and the ability of a drug that specifically targets phagocytic cells to indirectly or directly affect lymphocytes are important questions that must be answered before these studies can be regarded as therapeutic strategies.

Immunotoxins might provide a mechanism for targeted elimination of HIV-1-infected cells, and both novel design of immunotoxin-based antiviral agents and their effect on macrophages remains an ongoing area of research. Berger *et al.* [112] recently reported the use of *Pseudomonas aeruginosa* Exotoxin A linked to a specific protein that binds HIV-1 Env (3B3Mab against a highly conserved region of the $CD4^+$ binding site). Inhibition of spreading infection was observed in both primary lymphocytes and macrophages, and addition of an RT inhibitor eliminated infectious virus in lymphocyte cultures. Whether this agent can demonstrate antiviral activity in primary macrophages at different stages of activation and infection are not defined; however, these studies provide a foundation for further studies. One major limitation of this experimental protein is its inability to cross the BBB; thus, precluding its use as a monotherapy and instead limiting it to combination administration with other agents demonstrating greater CNS penetration.

Inhibitors targeting HIV-1 accessory proteins provide a targeted approach for elimination of HIV-1 within infected cells. Vpu provides an attractive target because interference with Vpu acts post-integration to confer abnormal packaging of newly formed virions. Khoury *et al.* [113] demonstrated that BIT225, an inhibitor of Vpu displays antiviral activity against an R5-using HIV-1 in primary macrophages during acute and chronic infection (EC_{50} 1,000 nM; Table 2). In addition, transfer of infection from primary macrophages to more permissive $CD4^+$ T-lymphocytes was also significantly reduced. These data provide the framework for a novel class of therapeutics targeting components of the virus that are essential for productive replication.

Cellular factors affecting pharmacology of ART in macrophages

Bioavailability of drugs is in the treatment of HIV-1 infection [56,106,114]. Many ARTs are highly plasma protein bound, metabolized by cytochromes in the intestinal epithelium or lack the lipophilicity to traverse the lipid bilayer of cells efficiently [106,114–116]. Some

drugs are substrates for efflux transporters, whose intrinsic function is to prevent cellular toxicity by performing rapid efflux of intracellular substrates. The link between efflux transporter expression and activity, and modulation of these transporters by ART has been extensively studied both *in vitro* and *in vivo*. To date, studies correlating higher efflux transporter expression and activity with treatment failure and poor clinical prognosis are conflicting [114,117,118].

P-glycoprotein (P-gp/MDR1) and multidrug resistance transporters 1, 4 and 5 (MRP1, MRP4 and MRP5) are expressed in macrophages [119–121]. As efflux transporters can limit the amount of bioavailable intracellular drug, understanding the link between these efflux transporters and bioavailability could provide a foundation for design of novel therapeutics that might not bind with high affinity to these transporters.

Relative to macrophages, efflux transporter expression and antiviral activity of AZT and IDV has been correlated in a study by Jorajuria *et al*. [119]. Inhibition of P-gp resulted in increased antiviral activity and increased intracellular IDV levels, whereas inhibition of P-gp did not correlate with increased anti-HIV activity of AZT. These data suggest that AZT is a substrate for MRP4 and MRP5, but not P-gp, whereas IDV is a substrate for P-gp.

Efflux transporters are a natural defence mechanism to prevent cellular toxicity and might decrease intracellular bioavailability of ART in HIV-1 target cells. Decreased bioavailability can result in suboptimal levels of drug, resulting in an increased likelihood for emergence of resistant HIV-1 [122]. As macrophages express P-gp, MRP4 and MRP5, and multiple ARTs have been demonstrated to be substrates for these transporters [119–121], it follows that this link might reduce the intracellular concentration of ART in macrophages. Long-term administration of ART could expedite emergence of resistant HIV-1 in macrophages, facilitating a viral reservoir for resistant HIV-1 within these long-lived cells. In addition, long-lived macrophages with suboptimal levels of drug might also remain latently infected, and emerge later in disease progression, transferring productive infection of resistant viruses [123]. In this way, the relationship between cellular factors affecting intracellular drug concentration directly correlates with latency, viral reservoirs and chronic HIV-1 infection. Understanding how current ARTs interact with efflux transporters and the effect of this relationship on therapy outcome and long-term patient survival is essential when designing novel therapeutics.

Conclusions

Macrophages represent a crucial target for establishment and maintenance of chronic and latent HIV-1 infection [2,3,21,31,33]. Various macrophage-like cells, including dendritic cells, alveolar macrophages, monocyte precursors and microglial cells, contribute to the complex interplay between systemic infection and administration of ART [2,3,16,21,22,25,26,31,33,73,84,87,124]. Macrophages are found in every organ system and tissue, and, because of high CCR5 expression, represent a target for early establishment and maintenance of latent viral reservoirs [2,4,7,71].

HIV-1 infection in alveolar macrophages can contribute to cytokine-mediated immune dysfunction by promoting a proinflammatory milieu that favours disease progression, whereas, in some cases, increasing levels of cytoprotective chaemokines including MIP1-α, MIP1-β and RANTES [76,125]. Infection in CD16hi HIV-1 permissive monocytes can contribute to increased trafficking of HIV-1-infected monocytes and macrophages across the BBB [86,87]. Increased trafficking across the BBB, in turn, positively modulates a proinflammatory cytokine milieu coupled with release of ROS in the brain, eventually culminating in an increased risk of HAD and MCMD [88,89,94].

Although the cellular pharmacology of ART remains an understudied area of research, the significantly higher EC_{50} in macrophages versus lymphocytes suggests that ART are not as potent across macrophages [126]. In addition, penetration of ART across the BBB is usually poor [57,95,96] and the ability to deliver effective concentrations of drug to macrophages across all organs and tissue compartments remains an important goal towards viral eradication. The cellular pharmacology of ART in macrophages directly affects viral loads, emergence of mutations that confer resistance both within and between subsets of HIV-1 target cells, eradication of systemic virus and long-term patient survival (Figure 1). Eradication of systemic HIV-1 infection is not possible without clearance of latently infected cells. For these reasons, understanding dynamics of ART pharmacology in macrophages and subsequently eliminating productive infection in these cells, is critical to eliminating systemic HIV-1 infection.

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Disclosure statement

RFS is a founder and major shareholder of Idenix Pharmaceuticals, Pharmasset Inc. and RFS Pharma. CG declares no competing interests.

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Annexure - 6

BEFORE THE OFFICE OF THE CONTROLLER OF PATENTS, NEW DELHI

IN THE MATTER OF:

The Patents Act, 1970 as amended by the Patents (Amendment) Act 2005, and The Patents Rules, 2003, as amended by The Patents (Amendment) Rules, 2016

AND

IN THE MATTER OF:

Indian Patent Application Indian Patent number 427560 (formerly patent application number 5348/DELNP/2014) filed by 1. EMORY UNIVERSITY having the principal business place at Office of Technology Transfer 1599 Clifton Road 4th Floor Atlanta GA 30322 2. GAVEGNANO, CHRISTINA having the principal business place at117 Mary Gay Cove, Decatur, GA 30033, United States of America 3. SCHINAZI, RAYMOND, F. having the principal business place at2881 Peachtree Road, NE, Unite 1403, Atlanta, GA 30305 (US).

AND

REPRESENTAION BY:

1. EMORY UNIVERSITY.....**APPLICANT/**
2. GAVEGNANO, CHRISTINA**PATENTEE**
3. SCHINAZI, RAYMOND, F.

vs

SANKALP REHABILITATION TRUST .....**OPPONENT**

AFFIDAVIT

[A] **INTRODUCTION:**

I, Mrs. Mansi Verma, age 39 years, D/o S S Verma residing at 116/3 Aditya World City Ghaziabad U.P. Pin Code 201002 and pursuing PhD from GLA University Mathura U.P., presently at Delhi do hereby state as under:

NOTARY SANYOGITA DELHI Regn. No. 7514 GOVT OF INDIA

1. I hold a Master's degree in the field of pharmacy with extensive experience in the field of pharmacology. I have over 11 years of experience after my Masters.

2. I have been approached by Sankalp Rehabilitation Trust (hereinafter referred as the "Opponent") regarding an Indian Patent number 427560, formerly patent application number 5348/DELNP/2014 (herein after referred as "impugned patent") in the name of1. EMORY UNIVERSITY2. GAVEGNANO, CHRISTINA and 3. SCHINAZI, RAYMOND, F (hereinafter referred as "Patentee/Applicant"). The relevant documents like complete specification, amended claims FER reply, written submission and other documents pertaining to the impugned patent was provided by Opponent. The impugned Patent covers and claims a composition comprising (a) JAK inhibitor(s) and (b) HAART agent(s). The claims are reproduced below for reference:

> 1. A composition for treating or preventing HIV comprising:
> a) an effective antiviral amount of a JAK inhibitor selected from the group consisting of CEP-701 (Lestaurtinib), AZD1480, LY3009104/INCB28050 Pacritinib/SB1518, VX-509, GLPG0634, INC424, R-348, CYT387, TG 10138, AEG 3482, 7-iodo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acryl amide, 7-(3-aminophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phen- yl)acrylamide, N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, methyl 2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxylate, N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(4-amino-3-methoxyphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 4-(2-(4-

morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, N,N-dimethyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 1-ethyl-3-(2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)urea, N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)metha- nesulfonamide, 2-methoxy-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)pheno- l, 2-cyano-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)phenyl)acetamide, N-(cyanomethyl)-2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidine-7-carboxamide, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 1-ethyl-3-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)-2-(-trifluoromethoxy)phenyl)urea 20. N-(3-nitrophenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(3-nitrophenyl)thieno[3,2-d]pyrimidin-2-amine, N1-(7-(2-ethylphenyl)thieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N1-(7-iodothieno[3,2-d]pyrimidin-2-yl)benzene-1,3-diamine, 7-(4-amino-3-(tri fluoromethoxy)phenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl-)acetamide, N-(cyanomethyl)-N-(3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(cyanomethyl)-N-(4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, N-(3-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)b- enzenesulfonamide, N-(4-(5-methyl-2-(4-morpholinophenylamino)-5H-pyrrolo[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-iodo-N-(4-morpholinophenyl)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, 7-(2-isopropylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine 40. N7-(2-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, N7-(4-isopropylphenyl)-N2-(4-morpholinophenyl)thieno[3,2-d]pyrimidine-2,7-diamine, 7-(5-amino-2-methylphenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, 7-iodo-N-(3-

morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine.. 7-(4-amino-3-nitrophenyl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-methoxypyridin-3-yl)-N-(4-morpholinophenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(7-iodothieno[3,2-d]pyrimidin-2-ylamino)phenyl)methanol, N-tert-butyl-3-(2-(3-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)be- nzenesulfonamide, N-tert-butyl-3-(2-(3-(hydroxymethyl)phenylamino)thieno[3,2-d]pyrimidin-7--yl)benzenesulfonamide, N-(4-morpholinophenyl)-7-(4-nitrophenylthio)-5H-pyrrolo[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(4-amino-3-nitrophenyl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine 53. N-(3,4-dimethoxyphenyl)-7-(2-methoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(3,4-dimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)b-enzenesulfonamide, 7-(2-aminopyrimidin-5-yl)-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,6-dimethoxypyridin-3-yl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-(2,4-dimethoxypyrimidin-5-yl)thieno[3,2-d]pyrimidin-2-amine, 7-iodo-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide,. 2-cyano-N-(4-methyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide.. ethyl 3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzoate, 7-bromo-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine,. N-(3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(cyanomethyl)-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl-)benzamide,. N-tert-butyl-3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, tert-butyl-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-pyrazole-1-carboxylate, 7-bromo-N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-tert-butyl-3-(2-(4-((4-ethylpiperazin-1-yl)methyl)phenylamino)thieno[3,- 2-d]pyrimidin-7-yl)benzenesulfonamide, N-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(cyanomethyl)-3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-

d]pyrimidin-7-yl)benzamide, N-tert-butyl-3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]- pyrimidin-7-yl)benzenesulfonamide, tert-butyl pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, 3-(2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzenesulfonamide, 7-(3-chloro-4-fluorophenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno- [3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(1-ethylpiperidin-4-yloxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl-)-1H-pyrazole-1-carboxylate, 7-(benzo[d][1,3]dioxol-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 5-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-1H-ind- ole-1-carboxylate, 7-(2-aminopyrimidin-5-yl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, tert-butyl 4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-5,6-di-hydropyridine-1(2H)-carboxylate, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)-phenyl)acetamide, N-(4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, N-(3-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanesulfonamide, 7-(4-(4-methylpiperazin-1-yl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno- [3,2-d]pyrimidin-2-amine, N-(2-methoxy-4-(2-(4-(morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)acetamide, 7-

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bromo-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, (3-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(3,4,5-trimethoxyphenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (3-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, (4-(2-(4-morpholinophenylamino)thieno[3,2-d]pyrimidin-7-yl)phenyl)methanol, N-(pyrrolidin-1-yl)ethoxy)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzyl)methanesulfonamide, tert-butyl morpholinomethyl)phenylamino)thieno[3,2-d]pyrimidin-7-yl)benzylcarbamate, N-(4-(morpholinomethyl)phenyl)-7-(3-(piperazin-1-yl)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(6-(2-morpholinoethylamino)pyridin-3-yl)-N-(3,4,5-trimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(2-ethylphenyl)-N-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)thieno[3,2-d]pyrimidin-2-amine, 7-(4-(aminomethyl)phenyl)-N-(4-(morpholinomethyl)phenyl)thieno[3,2-d]pyrimidin-2-amine, N-(4-(1-ethylpiperidin-4-yloxy)phenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-amine, N-(2,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, 7-bromo-N-(3,4-dimethoxyphenyl)thieno[3,2-d]pyrimidin-2-amine, N-(3,4-dimethoxyphenyl)-7-phenylthieno[3,2-d]pyrimidin-2-amine, and pharmaceutically acceptable salts and prodrugs thereof, and

b) highly active antiretroviral therapy (HAART) agent comprising:

a) at least one of an adenine, cytosine, thymidine, and guanine nucleoside antiviral agent, or

b) at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors.

2. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is zidovudine (AZT).

3. The composition of Claim 1, wherein the thymidine nucleoside antiretroviral agent is 2R,4R)-2-amino-9-[(2-hydroxymethyl)-I, 3-dioxolan-4-yl]adenine (DAPD) or (−)-β-D-2-aminopurine dioxolane (APD).

4. The composition of Claim 1, wherein the antiretroviral agents comprise:

a) cis-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ((-)-FTC) or (−)-cis-2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (3TC),

b) 9-[(R)-2[[bis[[isopropoxycarbonyl)oxy]-methoxy]-phosphinyl] methoxy] propyl] adenine fumarate (TDF),

c) DAPD or APD,

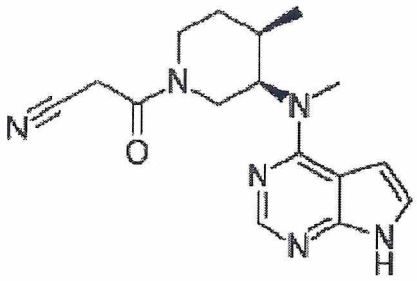
d) AZT, and

e) a NNRTI, a protease inhibitor, or an integrase inhibitor.

5. The composition of Claim 4, wherein the NNRTI is Sustiva, the protease inhibitor is Kaletra, or the integrase inhibitor is Raltegravir or Elvitegravir.

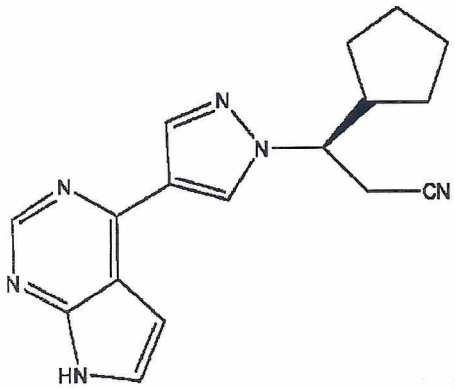
6. The composition of Claim 1, wherein the HAART agent(s) comprises at least one additional antiviral agent selected from the group consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, entry inhibitors, attachment inhibitors, and integrase inhibitors

7. The composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof.

8. The composition of Claim 1, wherein the JAK inhibitor compound is



, or a pharmaceutically acceptable salt or prodrug thereof. .

9. The composition of any of Claims 1-8, wherein the composition further comprises a macrophage depleting agent.

10. The composition of Claim 9, wherein the macrophage depleting agent is Boniva or Fosamax.

11. The composition of any of Claims 1-9, further comprising a reactivation agent.

12. The composition of Claim 11, wherein the reactivation agent is panobinostat.

3. I state that my Affidavit and that my opinion herein is purely based on my education, knowledge, training and experiences in the relevant scientific field.

4. I also want to declare that for consulting the matter described herein on this case, I am being compensated but neither is my compensation related to the outcome of these proceedings, nor do I have personal interest with bad faith in the instant matter.

5. Importantly, I am well aware that hindsight approach is not permissible in these deliberations. In addition to all of the above, I firmly believe that the inventions filed without merits should loudly be opposed.

6. I am no stranger to the requirements of an invention as per Indian Patents Act, 1970(herein after referred as "the Patents Act"). I am well aware that to be patentable,

the invention must be novel, inventive/non-obvious and must have industrial applicability. More importantly, the invention shall also cross the barrier of non-patentable subject matters that are described in the Patents Act.

7. As per the Patents Act, new invention means any invention or technology which has not been anticipated by publication in any document or used in the country or elsewhere in the world before the date of filing of patent application with complete specification, i.e. the subject matter has not fallen in public domain or that it does not form part of the state of the art.

8. I am quite acquainted with the definition of inventive step which is also known as non-obviousness and that it is a feature of an invention that involves technical advance as compared to the existing knowledge or having economic significance or both and that makes the invention not obvious to a person having ordinary skill in the art.

9. Thus, from above information, it may be clear to the Learned Controller that I am well mindful of Indian patents law also.

10. I am advised that this scrutiny is also to be done by a person skilled in the art who may be one with Masters Degree in (Pharmacology), and few years (10) of experience of working in research. I have also been exposed to patents and I am familiar with reading and understanding pharmaceutical patents as I have gone through the same in my career. Any 'skilled person' as described above is one who would be able to understand a patent and its working. I believe I would meet these criteria.

[B] EDUCATIONAL AND PROFESSIONAL BACKGROUND:

11. My educational qualifications, work experience, associations are more particularly stated in Exhibit-1, annexed hereto. In view thereof, I am the ordinarily person skilled in the art who is the addressee of the invention disclosed and claimed in the opposed patent application and I am competent to make this Affidavit, inter alia, expressing an opinion as an expert in the field of pharmaceutical sciences and pharmaceutical technology, on the issue stated herein below.

12. I deliberate on professional experience starting from present to old. Also, write about projects, student guided publications, etc. My previous work experiences are mentioned in detail in the form of my curriculum vitae enclosed herewith as Exhibit-1. A representative list of my career achievements is also attached herewith as Exhibit-1.

[C] TECHNICAL:

13. In view of my position, qualifications, experience and knowledge in the field of Pharmaceutical Sciences, I am competent to depose this Affidavit by expressing my opinions on the discussed matter.

14. I say that the claimed subject matter is obvious and devoid of any technical advancement in light of prior arts due to following reasons:

15. I say that HIV is a multifaceted disease that manifests itself over the course of several years and has multiple effects on the body of an affected individual that cause the quality of life of such an individual to deteriorate.

16. The HIV virus is known to affect several immune cells, including the $CD4^+$ T cells and macrophages. In an individual infected with HIV, lack of treatment leads to weakening

of the immune system as the immune cells like T cells progressively get depleted over the lifetime of an infected individual.

17. There has been a massive improvement in the treatments available for HIV, with the knowledge acquired as a result of research accomplished over the past several decades in discovering how HIV interacts with its host, the strategies it uses to evade the host response, and the pathways it uses in its lifecycle.

18. Based on this knowledge, several drugs have been developed targeting different pathways to disrupt the replication, assembly and other crucial events essential for the virus to survive and reproduce.

19. These drugs, although diverse insofar as the mechanisms they employ are concerned, are included in an umbrella term "HAART", which means highly active antiretroviral therapy.

20. HAART has been incredibly successful in controlling HIV in affected individuals and has thus greatly improved the quality of life of such individuals. HAART suffers from several drawbacks, however, such as the need to keep taking these drugs for the lifetime of the patient, toxicity in the long term, the struggle faced by affected individuals to stick to the required regimen, among others.

21. Researcher in this field over decades led to the discovery that even if HAART is able to keep viremia low, ultimately, a small number of viruses are able to survive and exist in certain sites, termed reservoirs. These sites can include, for example, $CD4^+$ T cells and macrophages. The virus is able to maintain a state of latency in such cells. Latency refers to a state where the virus is not actively dividing. However, this latent pool of

HIV represents a constant threat to someone who may discontinue the HAART regimen, as the virus can reactivate and cause rebound.

22. The success of HAART has encouraged researchers to contemplate that complete cure of HIV could be an achievable goal if the latent HIV could be eradicated. At the very least, a small of number of viruses existing in the body would mean that an individual can never completely go off HIV therapy.

23. With this background, I wish to provide my opinion of on the following prior art as discussed below in detail

i. WO2011109217A2 (WO'217) published 09 September 2011

ii. Richman et al., "The Challenge of Finding a Cure for HIV Infection"; Science, vol 323, published 6 March 2009

iii. Margolis David M., "Mechanisms of HIV Latency: an Emerging Picture of Complexity"; Curr HIV/AIDS Rep (2010) 7:37–43 published 27 January 2010

iv. Gavegnano et al., "Antiretroviral therapy in macrophages: implication for HIV eradication"; Antiviral Chemistry & Chemotherapy 2009 20: 63–78

24. I say that**WO2011109217A2** (WO'217) discloses methods of treating viral diseases that utilize RNA polymerase II for replication in the host. Specifically, the viruses HIV-1 and HIV-2, which also depend upon RNA polymerase are the focus of intervention in WO'217.

25. The pharmacological agent(s) disclosed in WO'217 for such targeting of RNA polymerase dependent viruses are (janus kinase 2) JAK2 inhibitors. Although WO'217 also discloses that tyrosine phosphorylation of HEXIM1 in the replication of RNA

polymerase dependent viruses, this is not the only mechanism by which a janus kinase inhibitor acts. The examples included in WO'217 for demonstration of activity (Example 1-2, internal page 49-54) relate to inhibition of HIV replication. Thus, actual demonstration of activity against HIV can be seen in WO'217. A person skilled in the art would, based on the above disclosure, strongly consider several janus kinase inhibitors for investigating their pharmacological activity against HIV. Thus, WO'217 provides a strong motivation to a person of ordinary skill in the art to investigate other effect of inhibiting the JAK also, with the goal of obtaining anti-HIV activity.

26. I say that Richman et al. is a discussion of what a strategy of completely eradicating HIV would entail. The limitations of HAART, despite its success, are discussed, as well as current known impediments involved in the complete eradication of HIV. The ability of HIV to go into a latent state is disclosed as an important impediment to such an effort.

27. It is disclosed that, although HAART has been incredibly successful in suppressing HIV replication below detection limits, low level viremia continues to persistin infected individuals. The virions produced in the body during such a process may ultimately cause cardiovascular and central nervous system disease. Richman et al. also highlights the concerns about potential long-term toxicity and prohibitive cost of HAART making it unsustainable on global scale (middle column, internal page 1304)

28. As a result of the above, Richman lays out that therapeutic treatments geared towards time-limited interventions that avoid resumption of viremia is a desirable goal, with the ideal being drug-free remission.

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29. Richman et al. discusses the sources of the low-level viremia in infected individuals who do not exhibit plasma viremia. These are latently infected and activated $CD4^+$ T cells and, potentially, lymphocytes (last column, page 1304). The other possible contributors to low-level viremia are chronically infected clonal reservoirs, monocytes and macrophages.

30. Referring to Fig. 2, it is disclosed that, Interleukin 7 (IL-7), a cytokine essential for maintenance of T cell homeostasis, can induce HIV expression from quiescent resting cells without global T cell activation, via the JAK/STAT5 signaling pathway. Thus, a JAK inhibitor would be expected to suppress HIV replication.

31. Richman et al. already disclosed (para 2, column 3, internal page 1304) that $CD4^+$ T cells are thought to be intermittently activated by antigen recognition or as bystanders in a local inflammatory process, which leads to the release of progeny virions. Thus, it was known that latently infected CD4+ T cells are involved in inflammatory processes.

32. Thus, to achieve HIV suppression and inflammation, administration of JAK inhibitor to target JAK/STAT5 signaling pathway would be a desirable option.

33. Richman et al. further discloses that the activation of latently infected cells in order to induce HIV expression can be achieved through various pathways. This activation would lead to lytic cell death of CD4+ T cells.

34. Thus, the goal is to induce the latently infected cell to be activated so that the virus is able to complete its replication cycle and exit its reservoir cell. After such exit, the released virions would inevitably attempt to infect new cells. In view of such a scenario, Richman et al. discloses that the strategy to eradicate infection would depend on current

or future antiretroviral therapy to completely inhibit all new infection events. (para bridging columns 1 & 2, internal page 1305).

35. Richman et al. discloses (Fig. 3, internal page 1307) administration of agents (termed "inductive agents") in combination with HAART to prevent further HIV spread to uninfected $CD4^+$ T cells. Thus, the strategy to combine two treatments, one of which can be used to induce the virus to replicate, and the other to target such viruses that have successfully replicated is suggested.

36. I say that Margolis David M. "Mechanisms of HIV Latency: an Emerging Picture of Complexity", discloses that despite the success of HAART in controlling HIV, currently, a cure has still not been achieved for it. It is further disclosed that clearance of HIV infection will almost certainly require a multimodality approach that includes potent suppression of HIV replication, therapies that reach all compartments of residual HIV replication, and depletion of any reservoirs of persistent, quiescent proviral infection (internal page 37).

37. It is further disclosed that viral persistence is maintained through T-cell survival and low-level antigen-driven proliferation and is slowly depleted with time in T_{CM} cells. In contrast, in aviremic patients with lower CD4+ counts and higher levels of interleukin (IL)-7-mediated homeostatic proliferation, proviral DNA is preferentially detected in T_{TM} cells. This finding suggests that IL-7-driven proliferation may result in host-driven replication of proviral genomes without the death of these infected cells, ensuring the persistence of this reservoir (internal page 41).

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38. Thus, Margolis highlights the role of central (T_{CM}) and transitional memory (T_{TM}) CD4+ T cells in the maintenance of HIV latency in infected individuals.

39. It is submitted that Gavegnano et al. discloses that macrophages infected with HIV are potential long-term HIV reservoirs that ought to be purged in order to achieve HIV eradication (Introduction, internal page 63).

40. Gavegnanofurther discloses the inability to eradicate systemic HIV-1 infection as a result of productive infection in brain microglia contributing to maintenance of an HIV-1 viral reservoirin the CNS. Resistant HIV-1 in the brain or CNS along with latently infected macrophages originating from other tissues and organs contributes to maintenance of macrophage viral reservoirs (internal page 64).

41. Thus, Gavegnano et al. discloses the macrophage as sites for maintenance of latent HIV in the body, and the role of such infected macrophages in effecting neurological impairment in HIV infected individuals. The need to target and eradicate this reservoir is disclosed.

42. Thus, WO'217, Richman et al., Argolis and Gavegnano, taken together, teach that HIV infection, whether active or that present in latently infected cells, can be targeted by using combination approaches. The latently infected cells can be CD4_ T cells, monocytes, macrophages and other cell types. Further, the utilization of janus kinase inhibitors, in combination with antiretroviral agents can target HIV in latent cells and represents path towards full eradication of HIV.

43. Thus, it is clear that by following the teaching of prior art WO'217, Richman et al., Margolis and Gavegnano et al. and common general knowledge a person skilled in the

art would arrive at the claimed composition in the Indian Patent 427560 (application 5348/DELNP/2014) which, therefore, is obvious.

Mansi

DEPONENT

VERIFICATION

I, Mansi Verma, the Deponent do hereby verify that the contents of my affidavit at para 1 to 43 are true and correct to the best of my knowledge, my experience and records. No part of it is false and nothing material has been concealed there from.

Verified at DELHI on this 21 day of DECEMBER, 2023

Mansi

DEPONENT

ATTESTED

NOTARY PUBLIC
DELHI (INDIA)

21 DEC 2023

NOTARY
SANYOGITA
Tis Hazari Courts
Delhi
Regn. No.

Annexure - 7

CURRICULUM VITAE

Mansi Verma
Mail: **mansi7184@gmail.com**
Mobile No:
+91-9711548505, 7439045807

Permanent address:

116/3, Aditiya World City, NH 24 Ghaziabad U.P. India

Local address:

116/3, Aditiya World City, NH 24 Ghaziabad U.P. India

Career Objective:

Having good academic qualification, industrial and academic experience, I wish to apply for the related vacancies in your organization. Where I can explore my abilities and knowledge for the development of the organization I work for and achieve the self projected goal **"to be a renowned and valuable employee in esteemed Institute".**

Academic Qualification:

YEAR OF PASSING	*SCHOOL/UNIVERSITY*	*QUALIFICATION*
2010	Shobhit University (Meerut)	Masters in Pharmacy (Pharmacology)
2008	MIET (Meerut)	B.Pharmacy
2002	C.B.S.E	10+2
2000	C.B.S.E	10th

M. Pharm Thesis

Title of the thesis: **"Antiulcer and Antioxidant activities of *Aegle marmelos* as experimental, biochemical and histopathology study"** under the supervision of **Dr. Ch.V Rao** Scientist, CSIR- National Botanical Research Institute, Lucknow, Govt. of India.

Strengths:

- Having strong academic record and work experience in industry and academics.
- Exposure in and always interested to update myself regarding the innovations in pharmaceuticals
- Ability to work in team.
- Deliver of work completion in time.
- Positive attitude and smart working ability.
- Hardworking nature and dedications to my work.

Work Experiences

Job Title: **Assistant Professor**
Year/months: From Oct 2010 to Oct 2012 (02 Years)
Employer: Teerthankar Mahaveer University**, City** Moradabad**, State:** U.P

Job Title: **Assistant Professor**
Year/months: From Jan 2013 to Jan 2014 (1Year)
Employer: Rao Neki Ram Memorial College of Pharmacy**, City** Gurgaon**, State:** U.P

Job Title: **Assistant Professor**
Year/months: From Jan 2014 to Feb 2015 (1Year)
Employer: S.D.College of Pharmacy and Vocational Studies.

Job Title: **Assistant Professor**
Year/months: From March 2015 to November 2019 (4 years 7month)
Employer: Meerut Institute of Engineering and Technology**, City** Meerut**, State:** U.P

Job Title: **Assistant Professor**
Year/months: From December 2020 to October 2021 (1Year)
Employer: Sunderdeep Pharmacy College**, City** Ghaziabad**, State:** U.P

Job Title: **Associate Professor**
Year/months: From October 2021 to Nov 2022 (1Year)
Employer: United College of Engineering & Research**, City** Greater Noida**, State:** U.P

Job Title: **Associate Professor**
Year/months: From November 2022 to September 2023 (10 months)
Employer: HR College of Pharmacy**, City** Ghaziabad**, State:** U.P

A patent application has been published in 2022, **application no: 202211071426**
Title:''Implementation of classification models and their impact on treating cancerous modes through nanoparticles."

Pursuing Ph.d from GLA University, Mathura.

Publications

1. Kumar A., Ashwlayan V. D., Verma, M., ―Investigation on Anti-ulcer activity of Hydroethanolic Flower Extract of Tagetes erecta L. on Swiss Albino Rats‖ **Journal of Ethnopharmacology** (Communicated Manuscript i. d. JEP_2020_1749).

2. Kumar, A. Ashwlayan, V D Verma M. ―Psychiatric drugs in medical setting: a review‖ Published in International Journal of Medical, Pharma and Research ISSN: 2456-8015 vol. 2 Issue 5 pp. 55-61 September 2018 **Impact Factor: 0.69**

3. Mansi Verma, Shyam Ji Gupta, Anurag Chaudhary, Vipin Kumar Garg; Protein tyrosine phosphatase 1B inhibitors as antidiabetic agents – A brief review; **Bioorganic Chemistry Volume 7**0, February 2017, Pages 267-283. **Impact Factor: 5.275** (**Scopus**)

4. Priyanka, Sangh Pratap, Mansi Verma, Keshari Kishore Jha" In vitro antioxidant activity of plant extract of Cressa Cretica" Scholars Research Library, **Der Pharmacia Lettre**, 2015, 7 (5):28-32. **(Impact factor 1.96)** (**Scopus**)

5. Ajeet, Mansi Verma, Sangeeta Rani and Arvind Kumar.''Antitarget Interaction, Acute Toxicity and Protein Binding Studies of Quinazolinedione Sulphonamides as GABA1 Antagonists", Published in **Indian journal of Pharmaceutical Sciences**, **Pubmed** ISSN: vol. 1 Issue 78, pp 48-53, 2016. **Impact Factor: 0.72** (**Scopus**).

6. .Kumar A., Ashwlayan, V. D., Verma, M. ―Diagnostic Approach & Pharmacological Treatment Regimen of Peptic Ulcer Disease‖ Published in Gastroenterology & Hepatology: vol.10 Issue 1, pp.33-41, January, 2019 DOI: 10.15406/ghoa.2019.10.00352.

7. Kumar A, Ashwlayan V D, Verma M., Garg V K Gupta SK Kumar A. Chaudhary A, Sharma A, Koul S, Kumar S Kumar A., Garg G., Sharma N Singh, L. Agrawal A, Soni S, Sharma, H.,Tyagi, H. Khan, F. ―A review on pharmacological treatment of sleep and circadian rhythm disorders‖ Sleep Medicine and Disorders: International Journal ISSN: 2577-8285 Vol. 3, Issue 1 pp.1–5 January 2019

8. Mansi Verma, Shyam Ji Gupta, Vipin Kumar Garg.,"Adverse Drug Reactions due to Systemic Medications- A Narrative Review", Published in International Journal of Pharmaceutical and Medicinal Research, ISSN: 2347-7008, vol. 3 Issue 4, pp 256-262, ,2015.

9. Ashok Kumar Gupta, Mansi Verma, Gajraj Singh Lodhi.," Protective effect of Ficus infectoria plant extract against fructose induced hyperlipidemia and hyperglycemia in wistar rats", Published in The Journal of Phytopharmacology, ISSN: 2230-480X, vol. 3 Issue 6, pp 431-435,2014.

10. Ajeet, Mansi Verma, Sangeeta Rani and Arvind Kumar.," In-Silico Designing And Screening of Quinazolinedione Sulfonamide Derivatives As Antibacterial Agents: A Docking Approach", Published in CIBTech Journal of Pharmaceutical Sciences, ISSN: 2319–3891, vol. 3 Issue 4, pp 20-31, 2014.

11. Ashok Kumar Gupta, Mansi Verma, Sawan Kumar, "Ficus infectoria shows protective effect against paracetamol induced liver damage in rats. Journal of Scientific and Innovative Research 2013, vol 6, issue 2, pp 1111-1115.

12. Sawan Kumar, K K Jha, Mansi Verma, Ashok Kumar, Dinesh Kumar.," Fructose induces Antihyperlipidemic activity of dried leaves extract of Alternanthera brasiliana L. Kuntz. in Wistar rats Published in The Journal of Phytopharmacology,
ISSN: 2320–480X, vol. 1 Issue 1, pp 71-81,2012.

13.Ashok Kumar, Mansi Verma, K. K. Jha, "Resealed Erythrocytes as a Carrier for Drug Targeting: A Review, Published in THE PHARMA INNOVATION, ISSN: 2277–7695, vol. 1 Issue 1, pp 7-15,2012

14. Mansi Verma.," A Review on Peptic ulcer: A Global Threat" Published in Journal of Pharmacy Research, ISSN: 0974-6943, vol. 3 Issue 9, pp 2088-2091, 2010.

15. Mansi Verma.," Investigation on Antiulcer Activity of Aegle marmelos Root as Experimental, Biochemical and Histological Study", Published in Journal of Pharmacy Research, ISSN: 0974-6943, vol. 3 Issue 10, pp 2088-2091, 2010.

16. Verma, M., Ashwlayan, V. D., Yadav, A. K. & Singh, R., ―Viscum album L- A Parasite Plant: A Review‖, Published in International Journal of Pharmaceutical Research and Development, ISSN: 0974–9446, vol. 2 Issue 4, pp 1-9, June, 2010 **(Impact factor 2.01).**

17. Amrish Kumar, Vrish D A, Mansi V. Disorder of Infancy and Childhood: A Review. Glob J Pharmaceu Sci. 2018; 5(5): 555675, page no: 001-005.

18. Kumar A, Ashwlayan VD, Verma M. Pharmacological strategies for the treatment of congestive heart failure. Pharm Pharmacol Int J. 2018;6(4):221–230, MedcAve publishers, page no: 001-007.

19. Kumar, A, Ashwlayan, V.D, Verma, M, et al., 2018. Investigation on Gastrointestinal Tract: A Review. *Lupine Online Journal of Medical Sciences,* Vol. 1, Issue 4, pp. 46-51.

20. Kumar, A, Ashwlayan, V.D, Verma, M, et al., 2018. Role of Chemical Entities in Ototoxicity. *Journal of Advanced Research in Medical Science & Technology*, Vol. 5, Issue 1, pp. 14-18.

21. Kumar A., Ashwlayan, V. D., Verma, M. Garg V. K. ―End Stage Renal Disease‖ Published in Journal of Advanced Scientific Research ISSN: 0976-9595 Vol.9, Issue 1 pp1-9 August 2018.

22. Kumar A., Ashwlayan V. D., Verma M., Garg, V. K., Gupta, S. K., ―A review on punctum plugs in the management of dry eye syndrome‖ Advances in Ophthalmology & Visual System ISSN: 2377-4290 vol. 8 Issue 4 pp. 255–257 August 2018 DOI:10.15406/aovs.2018.08.00316

23. Kumar A., Ashwlayan, V. D., Verma, M. ―Irritable Bowel Syndrome: A Review‖ published in Acta Scientific Pharmaceutical Sciences ISSN: 2581-5423 vol. 2, Issue 10, pp.14-18, September, 2018.

24. Kumar, A. Ashwlayan V.D., Mansi Verma, ―A Review on Drugs which Induce Liver Disease‖ Advances in Pharmacology & Clinical Trials ISSN: 2474-9214 vol. 3, Issue 2, pp. 000131, July 2018.

25. Kumar, A., Ashwlayan, V. D. Verma, M. ―Pharmacological strategies for the treatment of congestive heart failure‖ Published in Pharmacy & Pharmacology International Journal ISSN:

2373-6367 vol. 6, Issue 4, pp. 221-230, July 2018. DOI:10.15406/ppij.2018.06.00185

26. Kumar, A, Ashwlayan, V.D, Verma, M., et al., 2019. Role of *Camphlobacter pylori* and non steroidal anti-inflammatory drugs in the pathogenesis of peptic ulcer and pharmacological approaches for the treatment of peptic ulcer disease. *Gastroenterology & Hepatology: Open Access*, Vol. 10, Issue 1, pp. 33-41.

27. Kumar A., Ashwlayan, V. D., Verma, M. A review on pathophysiology, diagnosis and pharmacological management of septic colon Acta Scientific Pharmaceutical Science ISSN: 2581-5423 vol.3 Issue 2, pp.51-57, February, 2019.

28. Kumar A., Ashwlayan, V. D., Verma, M. ―Diagnostic Approach & Pharmacological Treatment Regimen of Peptic Ulcer Disease‖ Published in Gastroenterology & Hepatology: vol.10 Issue1, pp.33-41, January, 2019 DOI: 10.15406/ghoa.2019.10.00352.

29. Kumar A, Ashwlayan V D, Verma M., Garg V K Gupta SK Kumar A. Chaudhary A, Sharma A, Koul S, Kumar S Kumar A., Garg G., Sharma N Singh, L. Agrawal A, Soni S, Sharma, H., Tyagi, H. Khan, F. ―A review on pharmacological treatment of sleep and circadian rhythm disorders‖ Sleep Medicine and Disorders: International Journal ISSN: 2577-8285 Vol. 3, Issue 1 pp.1–5 January 2019.

Additional Qualification:

- Passed the WIPO (World Intellectual Property Rights Organization) exam in 2008.
- Self updating about the various pharmaceutical guidance and requirements.

Personal Information:

Date of birth : 7-01-1984
Nationality : Indian
Language known : Hindi & English (Spoken & Written)

DECLARATION:

I hereby declare the information furnished above is true to the best of my knowledge. That's all. Thanks for your precious time. I believe I am the right person for this post and with my knowledge and experience I will work for the benefit of the company.

Date: 21/Dec/23 **Mansi Verma**