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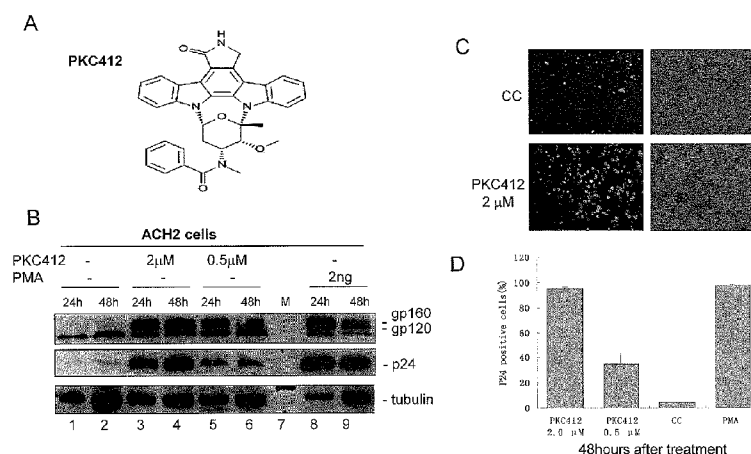


Fig. 1. Stimulation of HIV-1 expression in Latent infected ACH2 cells by PKC412.

(57) Abstract: HIV-1 latency is a major obstacle in HIV-1 eradication. Although current highly active antiretroviral therapy (HAART) has efficiently decreased the viral load in patients, latently infected cells are permanent reservoirs and inevitably lead to viral rebound upon discontinuation of HAART. Therefore, extensive efforts are being directed toward the reactivation of latent HIV reservoirs with the hope that latently infected cells will be eliminated by the host immune system and/or virus-mediated cell lysis. We are currently working on developing strategies to reactivate the latent viral reservoir in order to eliminate them. Through high-throughput screening, an anti-cancer molecule PKC412 that is able to reactivate HIV-1 expression from a HIV-1 latently infected ACH2 cell line and latently infected CD4⁺ T cells has been identified.

USE OF PKC412 IN THE REACTIVATION OF LATENT HIV-1

PRIOR APPLICATION INFORMATION

The instant application claims the benefit of US Provisional Patent Application
5 61/729,763, filed November 26, 2012.

BACKGROUND OF THE INVENTION

The major obstacle to HIV-1 eradication is the persistence of HIV-1 latency. Although anti-HIV chemotherapy has efficiently decreased the viral load in patients
10 (24), latent reservoirs persist and are irresponsive to immune surveillance and therapeutic treatment. Discontinuation of HAART often leads to the rapid rebound of the viral load (12, 29). Therefore, extensive research efforts have focused on finding ways to reactivate latent HIV-1 latent reservoirs and force them to be exposed to the host immune system for elimination.

HIV utilizes different strategies to survive in various cell reservoirs for persistent infection. Resting CD4+ T cells are the major cellular reservoir for viral persistence, including both naïve and memory CD4+ T cells (7, 10, 20). Other cell types that can be infected latently and contribute to viral persistence include peripheral blood monocytes (PBMCs), dendritic cells and macrophages (1, 2, 8, 13,
20 30). Circulating monocytes migrate to peripheral sites and differentiate into macrophages, which are responsible for viral spread and cell-to-cell transmission. Two forms of viral latency, preintegration and postintegration latency, have been described based on whether the virus integrates into the host genome (see reviews (5, 19)). Postintegration latency is of clinical significance because of its long-term
25 persistence in latently infected CD4+ T-cell reservoirs.

To eradicate HIV-1 latency, HAART intensification has been tested. However, HAART intensification failed to clear residual viremia with the addition of an additional potent ARV drug (9). Another strategy to target and clear the latent reservoirs is to reactivate HIV-1 latency in patients on HAART. The reactivated cells would be killed
30 by the cytopathic effects of the virus or by HIV-1-specific cytolytic T lymphocytes (6,

23, 26). Several different agents/compounds, such as immune activators, histone deacetylase inhibitors (HDACi) such as vorinostat and cell signal pathway modulators, are being explored. Most profoundly, various HDACi have been successfully used to stimulate latent virus production (3, 14, 15, 18). Furthermore, a number of PKC agonists, including PMA and prostratin (4, 16, 17, 21, 28), have been shown to induce HIV-1 transcription in latently infected CD4+ T cells or in PBMCs from HAART-treated patients. All of these findings strongly support reactivating HIV-1 latently infected cells as a promising approach for the targeting of HIV-1 latency.

10 SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method of inducing HIV expression in an individual in need of such treatment comprising administering to said individual an effective amount of PKC412.

15 According to a second aspect of the invention, there is provided a method of treating an HIV infection comprising: administering to an individual who is undergoing another treatment for HIV infection an effective amount of PKC412.

According to a third aspect of the invention, there is provided a method of preparing a medicament for inducing HIV expression in an individual in need of such treatment comprising admixing an effective amount of PKC412 with a suitable
20 pharmaceutically acceptable excipient or carrier.

According to a fourth aspect of the invention, there is provided the use of PKC412A for inducing HIV expression in an individual.

According to a fifth aspect of the invention, there is provided the use of PKC412A in treating an HIV infection as part of a highly active antiretroviral therapy
25 treatment.

According to a sixth aspect of the invention, there is provided the use of PKC412 in preparing a medicament for inducing HIV expression.

BRIEF DESCRIPTION OF THE DRAWINGS

30 **Fig. 1. Stimulation of HIV-1 expression in Latent infected ACH2 cells by**

PKC412. **A)** PKC412 (Midostaurin) is a multi-target protein kinase inhibitor that has successfully finished Phase II clinical trials for the treatment of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). **B)** HIV-1 latently infected ACH2 cells were cultured in the presence of PKC (0.5 or 2 μ M) and PMA (2ng/ml). The latter is used as a positive control. After 24 and 48 hrs, cells were lysed and analyzed in SDS-PAGE followed by Western blot with anti-HIV gp120, anti-HIVp24 and anti-tubulin antibodies. **C)** After 24 hrs, cells were fixed with acetone/methanol and labeled with anti-HIVp24 antibody/anti-mouse IgG-FITC antibody and visualized under fluorescence microscope. The percentage of HIVp24 positive cells was also determined (D).

Fig. 2. Pulse treatment of PKC412 stimulates HIV-1 expression in ACH2 cells. **A)** ACH2 cells were pulse-treated with PKC412 (1 μ M) for 4, 8, 12, 16, 24 hrs and the compound was washed away. Cells continued to be cultured up to 48 hours. As positive controls, cells were treated with PKC412 (1 μ M) and PMA (2 ng/ml) for 48 hrs. After 48 hrs, cells were collected and viable cells counted (A), as well as the percentage of HIVp24-positive cells (B). Supernatants were collected for p24 assay using HIV-1 p24 ELISA kit for determining HIVp24 levels (C), and equal amounts of cells were lysed and analyzed by western blot with anti-HIV gp120, anti-HIVp24 and anti-tubulin antibodies (D).

Fig. 3. PKC412 stimulates HIV-1 expression in serum starvation-induced quiescent ACH2 cells. ACH2 cells were cultured in RPMI medium (0.5 % FBS) for two days. Cells were treated with different concentrations (0.25, 0.5 and 1.0 μ M) of PKC412 and cultured in serum starvation medium. At different days as indicated, supernatants were collected and measured for HIVp24 production (A). After 48 hrs of PKC412 treatment, cells were harvested and analyzed by SDS-PAGE followed by Western blot with anti-HIV gp120, anti-HIVp24 antibody (B). 10% of the cell population were fixed with acetone/methanol and labeled with anti-HIVp24 antibody/anti-mouse IgG-FITC antibody and visualized under a fluorescence microscope.

Fig. 4. Synergistic reaction of latent HIV-1 expression by PKC412 with Vorinostat. ACH2 cells were treated with PKC412 and/or vorinostat (VOR) at different concentrations. The effect of synergistic activation of HIV expression was determined by measuring HIV p24 level in supernatants 48 hours after treatment (A).
5 A WST assay was performed to determine the growth of cell populations at 2 after treatment with PKC and/or VOR (B).

Fig. 5. Establishing postintegration HIV-1 latency in primary CD4+ T cells and reactivating virus by drugs. (A) An experimental schematic summary. Purified CD4+ T cells were infected by HIV-1 by spinoculation at 1200g for 2 hrs, then cultured
10 in medium without activation. 3 days later, cells were stimulated with PKC412 and/or vorinostat. (B) A representative experiment shows the levels of p24 in the supernatants of latent infected resting CD4+ T cells treated with or without PKC412 and/or vorinostat for 2 days.

Fig. 6. PKC412 does not affect histone acetylation. (A) Western blot
15 detection of acetylated histone H3 levels in latently infected cells. ACH2 cells were mock treated or treated with PKC412, butyrate or vorinostat and cells were collected after different times. Western blot analysis was performed with anti-acety-histone H3 and anti-histone H3 antibodies. (B) Diagram shows the positions of nucleosomes bound to the HIV-1 LTR and the location of a primer used for real time PCT ChIP
20 assay. (C) Cells treated with or without PKC412 were assayed by ChIP with anti-acety-histone H3, while immunoprecipitation with IgG serves as a negative control. Cells treated with butyrate were used as a positive control. Real-time quantitation of the fold change relative to negative control is shown.

25 DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention,
30 the preferred methods and materials are now described. All publications mentioned

hereunder are incorporated herein by reference.

HIV-1 latency is a major obstacle in HIV-1 eradication. Although current highly active antiretroviral therapy (HAART) has efficiently decreased the viral load in patients, latently infected cells are permanent reservoirs and inevitably lead to viral rebound upon discontinuation of HAART. Therefore, extensive efforts are being directed toward the reactivation of latent HIV reservoirs with the hope that latently infected cells will be eliminated by the host immune system and/or virus-mediated cell lysis. The inventors are currently working on developing strategies to reactivate the latent viral reservoir in order to eliminate them. Through high-throughput screening, the inventors have identified an anti-cancer molecule PKC412 that is able to reactivate HIV-1 expression from an HIV-1 latently infected ACH2 cell line. Herein, the inventors further demonstrate its anti-latency effect in primary HIV-1 latently infected CD4⁺ T lymphocytes. This demonstrates the activity of PKC412 as a new anti-HIV latency agent suitable for contributing to HIV-1 eradication.

Typically, HAART treatment is a combination of two or more of: nucleoside analogs; nonnucleoside reverse transcriptase inhibitors; protease inhibitors; fusion and entry inhibitors and integrase inhibitors.

Accordingly, in one aspect of the invention, there is provided a method of inducing HIV expression in an individual in need of such treatment comprising administering to said individual an effective amount of PKC412.

In a further aspect of this embodiment, vorinostat is coadministered with PKC412.

As will be appreciated by one of skill in the art, "co-administration" does not necessarily mean that vorinostat is administered at exactly the same time as with PKC412. While the two compounds may be co-administered in one dose, they may be coadministered in separate dosage units within a suitable time frame for effective treatment.

As will be appreciated by one of skill in the art, determination of an "effective amount" of PKC412 that is sufficient to induce expression of latent HIV viruses can be determined through routine experimentation and will depend on several factors, for

example, the age, weight and general condition of the patient as well as the severity of the infection. Specifically, an effective amount will be an amount that is sufficient to stimulate HIV expression. For example, an effective amount may be an amount of PKC412 that is able to mimic the effective range determined in cell culture *in vivo*, that is, a concentration of between 0.06 μM to 6 μM .

According to another aspect of the invention, there is provided a method of treating an HIV infection comprising: administering to an individual who is undergoing another treatment for HIV infection an effective amount of PKC412.

In a further aspect of this embodiment, vorinostat is coadministered with PKC412.

In these embodiments, the administration of PKC412 is effectively part of the highly-active anti-retroviral therapy. As such, PKC412 will make the highly-active anti-retroviral therapy more effective by inducing expression of HIV, especially HIV viruses that would otherwise remain dormant or latent during the HAART process. As a consequence, these once-latent viruses are not available for reactivation once the HAART process is discontinued, meaning that there will be no rapid rebound of viral load.

According to another aspect of the invention, there is provided a method of preparing a medicament for inducing HIV expression in an individual in need of such treatment comprising admixing an effective amount of PKC412 with a suitable pharmaceutically acceptable excipient or carrier. As will be appreciated by one of skill in the art, in these embodiments, an "individual in need of such treatment" is an individual who has an HIV infection and is undergoing therapy or treatment for this infection, for example, highly active anti-retroviral therapy.

In a further aspect of this embodiment, PKC412 may be formulated for coadministration with vorinostat.

In other embodiments, there is provided a method of preparing a medicament for inducing HIV expression in an individual in need of such treatment comprising admixing PKC412 and vorinostat.

Accordingly, in yet other aspects of the invention, there is provided the use of

PKC412 for inducing HIV expression in an individual, for treating an HIV infection as part of a highly active antiretroviral therapy treatment and in preparing a medicament for inducing HIV expression.

As discussed herein, in some embodiments, PKC412 is used with vorinostat
5 for inducing HIV expression in an individual.

As discussed herein, PKC412 is a multi-target protein kinase inhibitor and has been used to treat acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) successfully. Consequently, it is very surprising that it can also be used to induce HIV expression. For example, one other agent known to disrupt HIV-1 latency
10 is vorinostat is a histone deacetylase inhibitor, not a protein kinase inhibitor. Furthermore, as shown in Figures 4 and 6 and as discussed below, vorinostat and PKC 412 act through different mechanisms. Furthermore, while induction of HIV expression would be detrimental to some patients undergoing treatment for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), the inventors have
15 realized how this compound can be used beneficially to overcome the significant problem of latent HIV infections avoiding HAART therapies.

As discussed above, the inventors conducted a high throughput screening to find new small molecule that could activate HIV-1 expression in HIV-1 latently infected T cells. Through the screening, the inventors have demonstrated that a molecule
20 called PKC412 (Midostaurin) (Fig. 1A) (see Published PCT Application WO2011/064355, the contents of which are incorporated herein by reference for the disclosures on the synthesis of PKC412 and related compounds) that has been successfully completed phase IIB clinical trials for treatment of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (11, 22, 25, 27), is able to
25 increase HIV-1 gene expression in HIV-1 latently infected ACH2 cells. When ACH2 cells were cultured in the presence of PKC412 (0.5 or 2 μ M) for 24 and 48 hrs, the expression of HIV-1 Ggap24 and envelope glycoprotein were induced to significantly higher levels compared to that in non-treated cells, based on detection of HIV-1 protein expression in the cells by using SDS-PAGE followed by Western blot analysis
30 with anti-HIV gp120 and anti-HIVp24 antibodies (Fig. 1B). After 24 hrs of treatment,

the percentage of HIVp24 positive cells was counted after cells were fixed with acetone/methanol, labeled with anti-HIVp24 antibody/anti--mouse IgG-FITC antibody and visualized under fluorescence microscope. Results have clearly shown that a significantly higher percentage of ACH2 cells were induced to express high levels of HIV-1 proteins (Fig. 1C and D). All of these data clearly indicate that PKC412 reactivates HIV-1 latently infected cells by stimulating HIV expression.

The inventors then determined whether the persistent presence of PKC412 is required for its stimulating effect on HIV-1 expression. To answer this question, the inventors pulse-treated ACH2 cells with PKC412 (1 μ M) for 4, 8, 12, 16, 24 hrs, and then the compound was washed away and cells were cultured up to 48 hours. As positive controls, cells were treated with PKC412 (1 μ M) or PMA (2 ng/ml) for 48 hrs. After 48 hrs, cells were collected and counted for viable cells and the percentage of HIVp24-positive cells (Fig. 2A and B). HIV-1 expression levels released in the supernatant and in the cells were measured using HIV-1 p24 ELISA kit (Fig. 2C) and Western blot analysis with anti-HIV gp120 and anti-HIVp24 antibodies (Fig. 2D). Results showed that when cells were treated with PKC412 for 8 hrs, the amounts of viable cells were reduced about 16% (Fig. 2A). However, the HIVp24 positive cells were increased 5.2-fold (Fig. 2B) and the virus production in the supernatant was 4-fold higher than that in the absence of PKC412 (Fig. 2C). When cells were treated with PCK412 for 12 hrs, more increased levels of HIV-1 expression in both cells and in the supernatant were observed (Fig. B to D). All of these results demonstrate that a short time (8-12 hrs) exposure of ACH2 cells to PKC412 is sufficient to significantly stimulate HIV-1 expression.

The persistence of HIV-1 in non-proliferating quiescent T cells and monocytes is a major obstacle for the eradication and cure of HIV infection. The results discussed above demonstrate that PKC412 is able to reactivate gene expression in proliferating ACH2 cells.

However, whether PKC412 is also able to activate HIV-1 expression in quiescent HIV-infected T cells was unknown. To test this possibility, the inventors cultured HIV-1 latently infected ACH2 cells in serum-starved (0.5% serum) medium

for 48 hrs to arrest cells in the G0 phase. Then, cells in starvation medium were treated with various concentration of PKC412 for 24 hrs, washed, and continued to be cultured at 37°C in serum-starved medium for another 24 hrs. Finally, the cells were collected, and HIV p24 levels in the cells and in the supernatant were measured by anti-HIV p24 ELISA (Fig. 3A) and anti-HIV p24 Western blotting or indirect fluorescence assays (Fig. 3B and 3C). These results clearly showed that PKC412, at concentration of 0.25 to 1 ug/ml, stimulated HIV expression in quiescent ACH2 cells (Fig. 3A-C). From these results, it is clear that PKC412 is able to reactivate HIV-1 expression not only in proliferating ACH2 cells, but also in quiescent ACH2 cells under serum starvation condition.

As discussed above, vorinostat is a histone deacetylase inhibitor that is known to disrupt HIV-1 latency.

As discussed above and as shown in Figure 4, comparative concentrations of PKC412 induced HIV-1 P24 expression in ACH2 cells to a greater extent than VOR. Furthermore, the combination of VOR and PKC412 induces HIV-1 P24 expression to a much greater extent than either PKC412 or VOR, as shown in Figure 4A.

As shown in Figure 5B, treatment with either vorinostat or PKC412 induced HIV-1 P24 expression in latently-infected CD4+ T cells. Furthermore, the combination of vorinostat and PKC412 showed an additive effect in the induction of HIV-1 P24 expression, clearly demonstrating that each compound induces HIV-1 expression from latency by a different mechanism.

This is confirmed in Figure 6 which demonstrates that treatment with PKC412 did not inhibit histone deacetylation as was the case with vorinostat and butyrate.

In conclusion, the inventors' current research has shown that PKC412 is able to stimulate HIV-1 gene expression in HIV-1 latently infected ACH2 cells and CD4+ T cells. PKC412 acts through a different mechanism than vorinostat and is as effective at inducing HIV expression. Furthermore, the two compounds, when used in tandem, surprising induce HIV expression to a much greater extent than either alone.

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CLAIMS

1. A method of inducing HIV expression in an individual in need of such treatment comprising administering to said individual an effective amount of PKC412.

5 2. The method according to claim 1 wherein said individual is co-administered an effective amount of vorinostat.

3. A method of treating an HIV infection comprising:
administering to an individual who is undergoing another treatment for HIV infection an effective amount of PKC412.

10 4. The method according to claim 3 wherein the individual is co-administered an effective amount of vorinostat.

5. The method according to claim 3 wherein the treatment for HIV infection is a highly active antiretroviral therapy treatment.

15 6. A method of preparing a medicament for inducing HIV expression in an individual in need of such treatment comprising admixing an effective amount of PKC412 with a suitable pharmaceutically acceptable excipient or carrier.

7. The method according to claim 6 wherein the PKC412 is formulated for coadministration with vorinostat.

8. Use of PKC412A for inducing HIV expression in an individual.

20 9. Use of PKC412A in treating an HIV infection as part of a highly active antiretroviral therapy treatment.

10. Use of PKC412 in preparing a medicament for inducing HIV expression.

25 11. The use according to any one of claims 8-10 wherein the PKC412 is used with vorinostat.

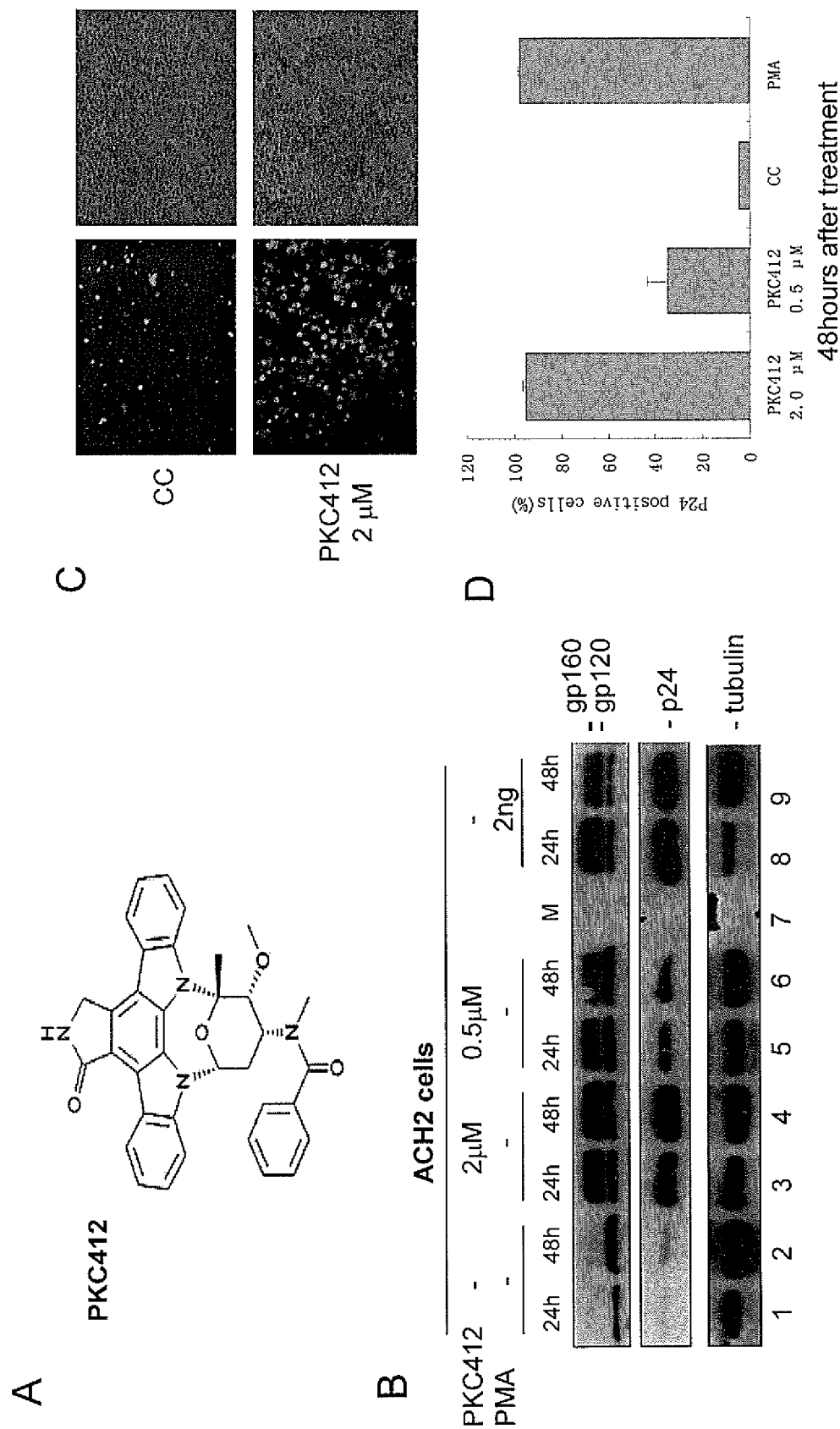


Fig. 1. Stimulation of HIV-1 expression in Latent infected ACH2 cells by PKC412.

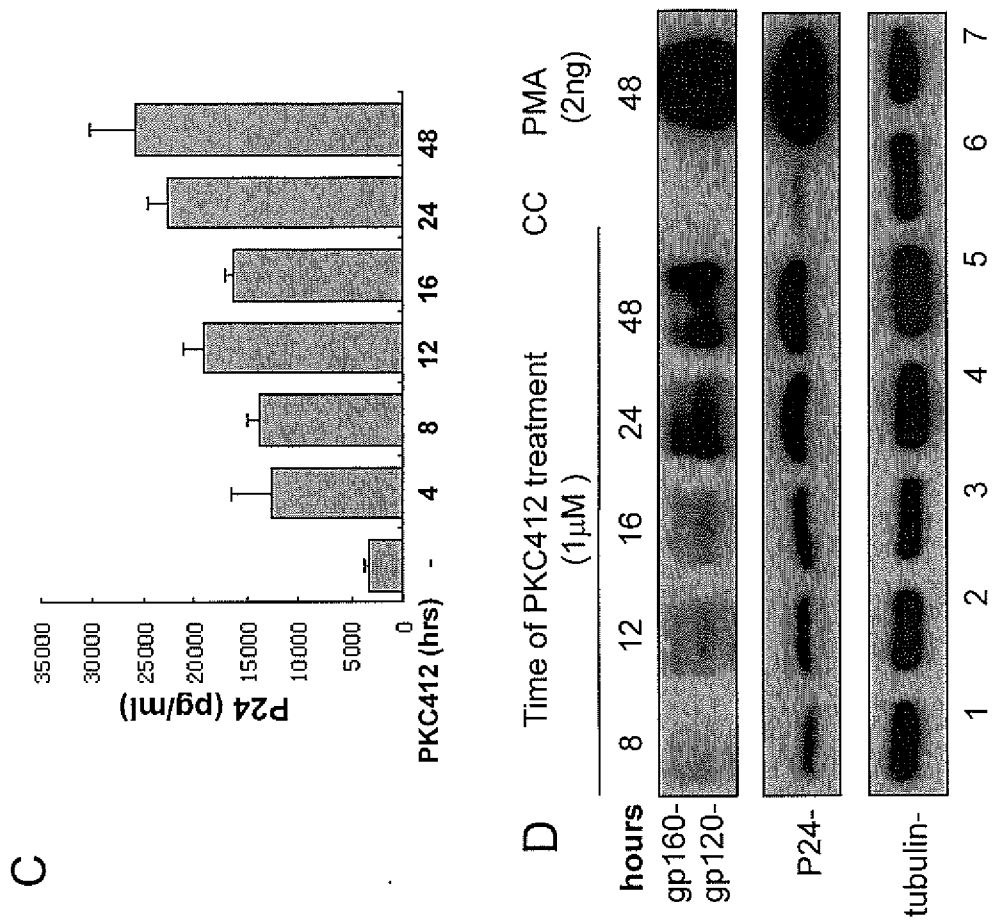
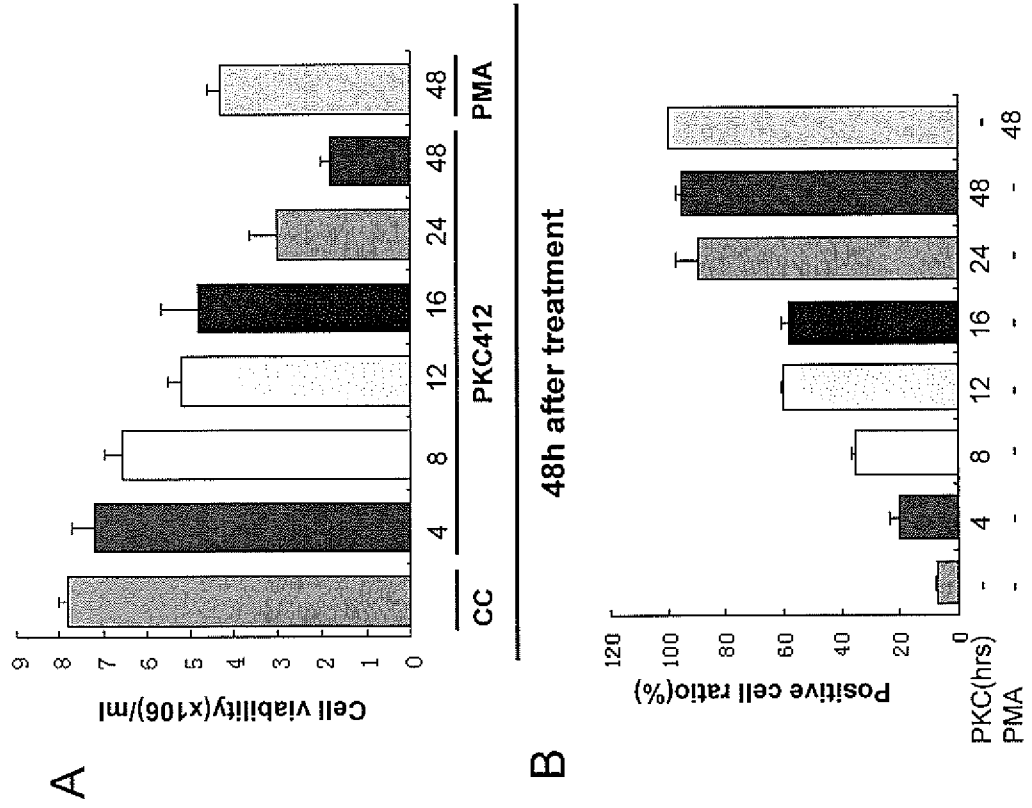


Fig. 2. Pulse treatment of PKC412 stimulates HIV-1 expression in ACH2 cells.

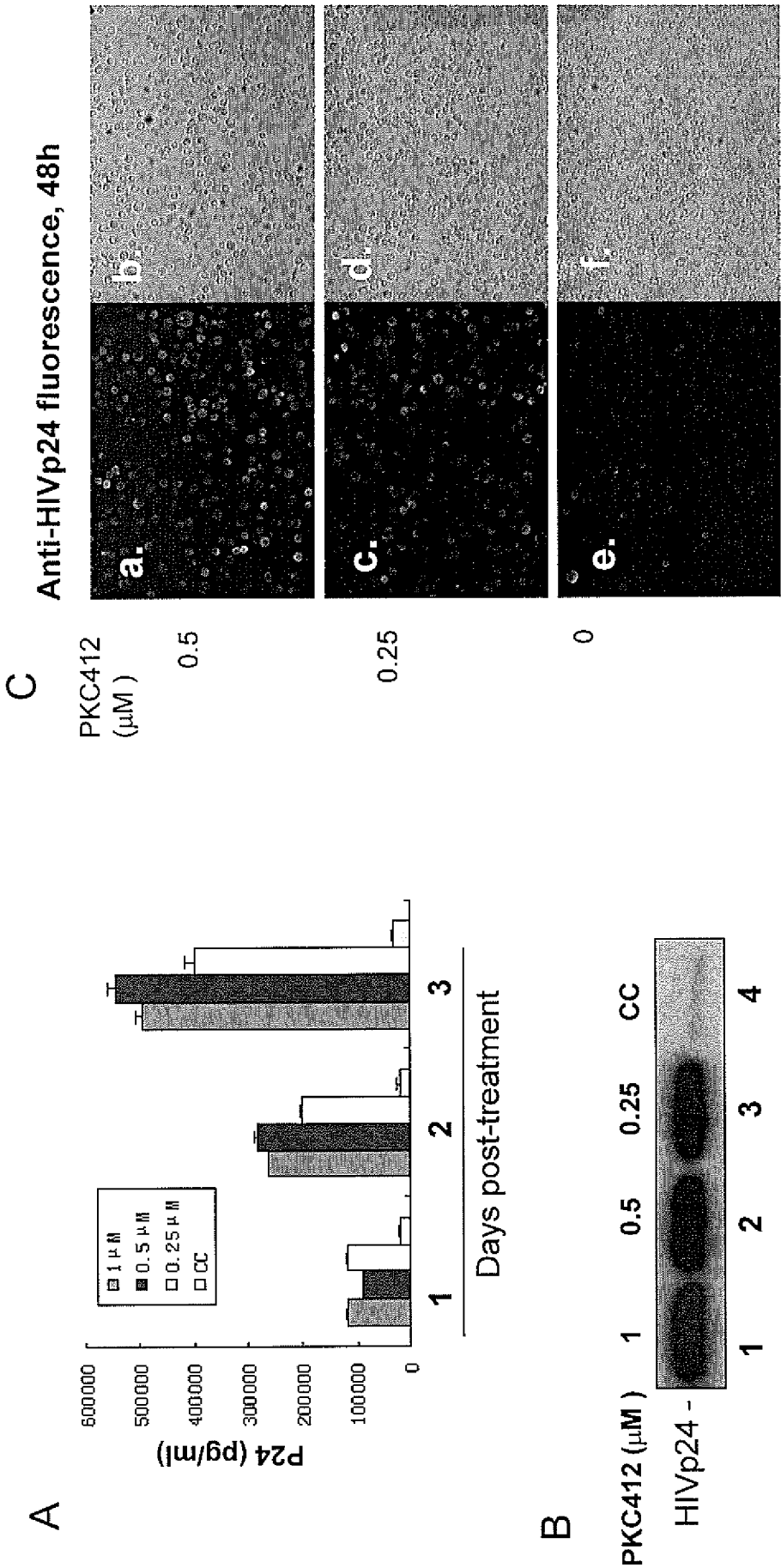


Fig. 3. PKC412 stimulates HIV-1 expression in serum starvation-induced quiescent ACH2 cells.

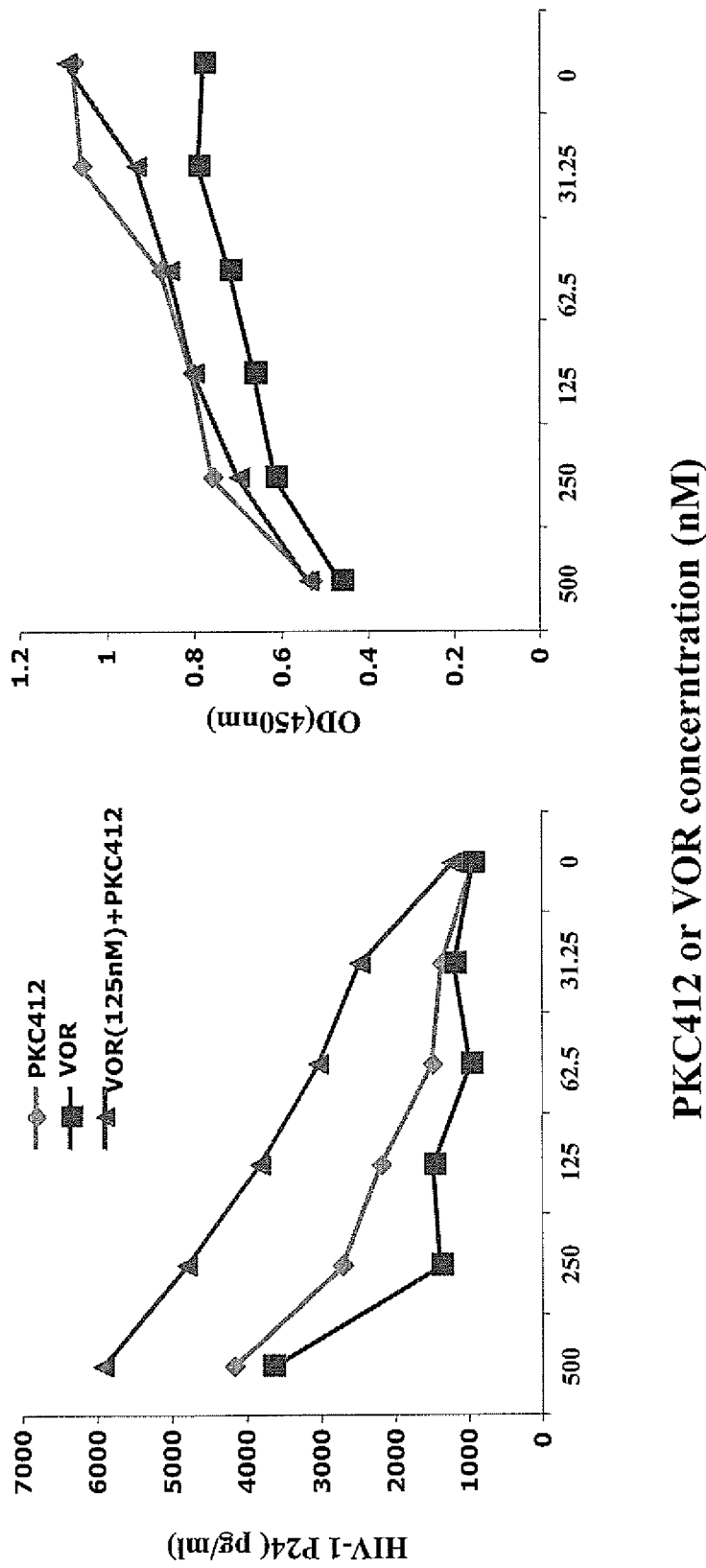


Fig.4. Synergistic reaction of latent HIV-1expression by PKC412 combination with Vorinostat.

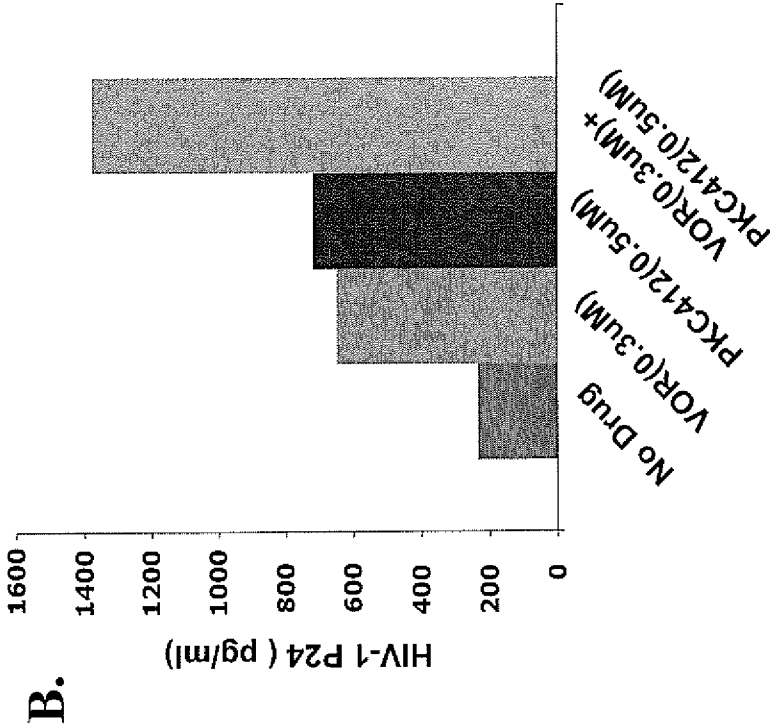
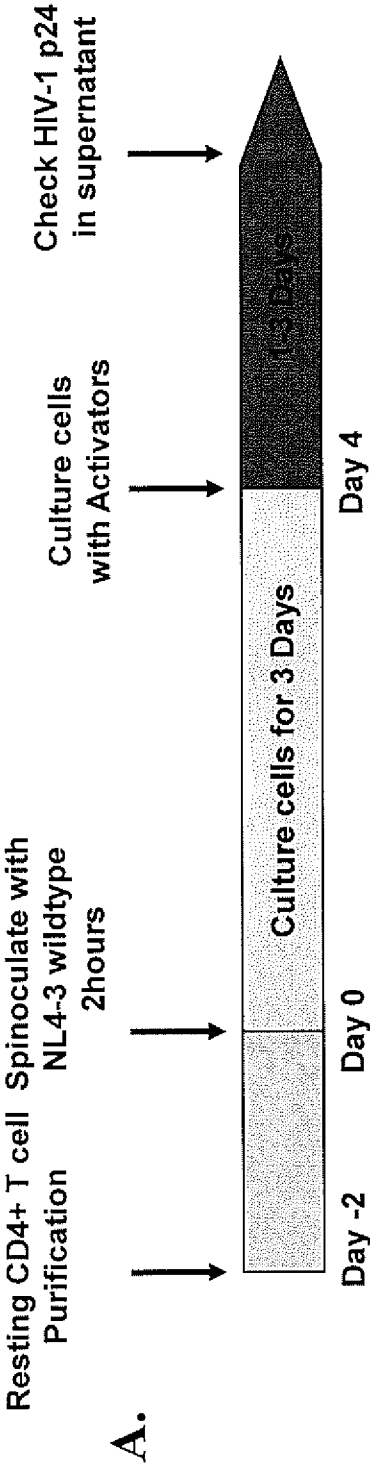


Fig.5. Establishing post-integration HIV-1 latency in primary CD4+ T cells and reactivating virus by drugs.

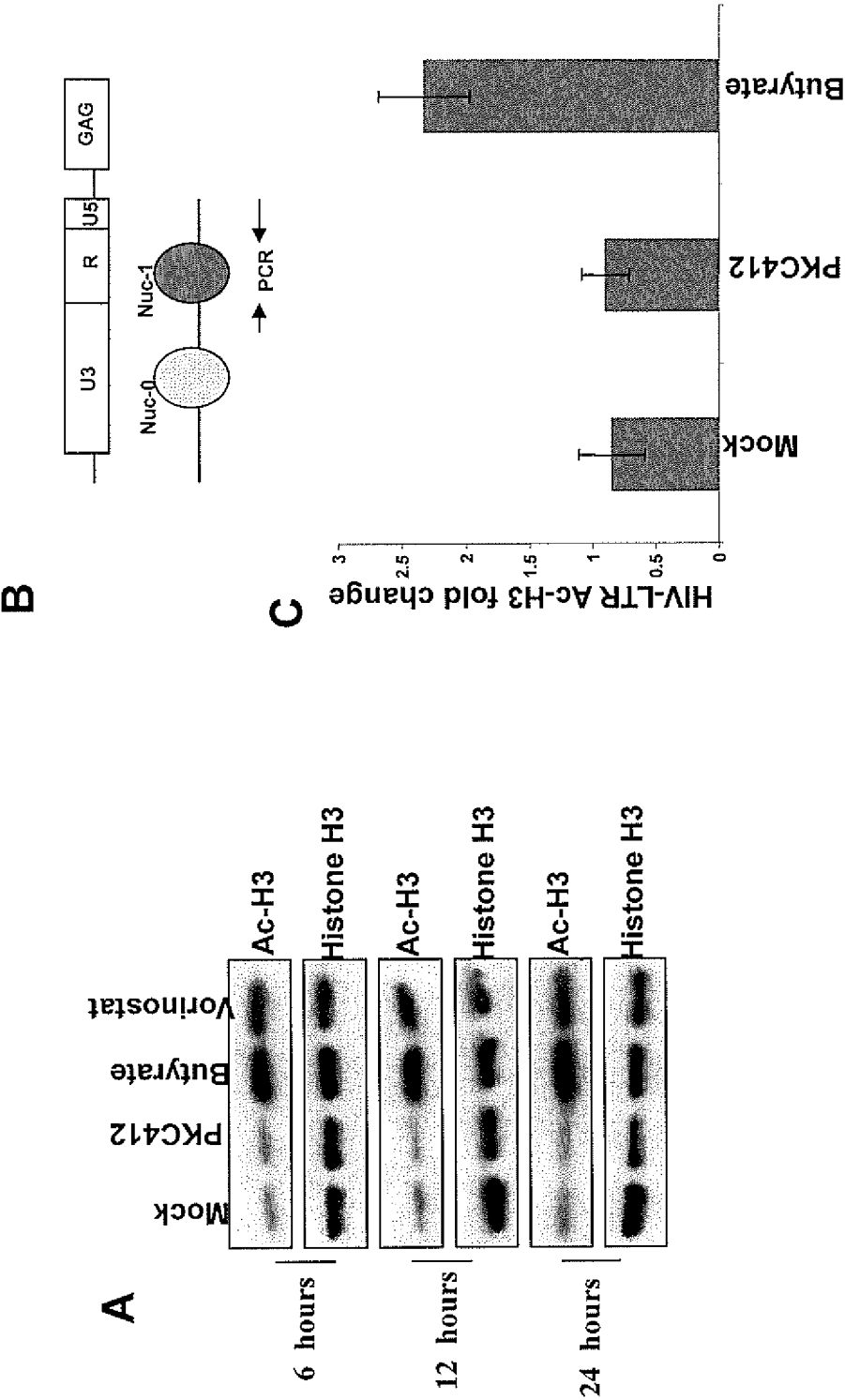


Fig. 6. PKC412 does not affect histone acetylation.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2013/050898

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 31/553** (2006.01), **A61K 31/167** (2006.01), **A61P 31/18** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 31/553 (2006.01), **A61K 31/167** (2006.01), **A61P 31/18** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: Canadian Patent database (Intellect), PubMed, TotalPatent, Google Scholar**Keywords:** PKC?412, Midostaurin*, staurosporine, Vorinostat, HDACi, HIV, latent, induc*

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patzold, et al. "Novel indolocarbazole protein kinase C inhibitors prevent reactivation of HIV-1 in latently infected cells", Antiviral Research, 1993, Vol 22, pages 273-283.	6-11
A	Kulkosky, et al. "Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART", Blood, 2001, Vol 98, pages 3006-3015.	6-11

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
18 March 2014 (18-03-2014)Date of mailing of the international search report
28 March 2014 (28-03-2014)Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2013/050898

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 1-5

Claims 1-5 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 1-5.

2. ☐ Claim Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.