Inhibition of HIV-1 Nef-induced apoptosis of uninfected human blood cells by serine/threonine protein kinase inhibitors, fasudil hydrochloride and M3

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Abstract The Nef protein of HIV-1 binds to and induces apoptotic cytolysis of uninfected but activated human peripheral blood mononuclear cells (PBMC) and various cell line cells derived from CD4$^+$ T, CD8$^+$ T and B lymphocytes, macrophages, and neutrophils. The Nef-induced apoptosis also occurs with blood cells not expressing CD95 (Fas). The Nef-induced apoptosis as well as Fas-mediated apoptosis was inhibited by acetyl-Try-Val-Ala-Asp-CHO, an IL-1β converting enzyme (ICE) inhibitor. On the other hand, serine/threonine protein kinase (PK) inhibitors, H-7, fasudil hydrochloride and M3, inhibited the Nef-induced apoptosis, and not the Fas-mediated one, without affecting the cell-binding activity of Nef and Nef-binding capacity of the activated cells. Preincubation of the cells with the drugs before being bound by Nef was required for the inhibition of apoptosis. These results suggest that the PK inhibitors specifically act on a cellular protein involved in the upper stream of signal transduction pathway of the Nef-induced apoptosis, which is different from the Fas-mediated pathway but meets it upstream of ICE. In addition, the drugs suppressed the cellular activation-associated cell surface expression of a putative Nef-binding protein in PBMC, although they had no influence on its expression in cell line cells. These findings suggest the feasibility of clinical use of the PK inhibitors to prevent the development of AIDS by inhibiting the Nef-induced apoptosis of uninfected blood cells.

Key words: AIDS; HIV-1; Nef; Apoptosis; Protein kinase inhibitor

1. Introduction

During the subclinical stage of HIV-1 infection, a variety of uninfected blood cells including CD4$^+$ T and CD8$^+$ T lymphocytes and neutrophils are destroyed more extensively showing a rapid turnover than virus-infected CD4$^+$ T cells and macrophages [1–3]. Extended death of the uninfected cells should result in immune suppression leading to the development of AIDS [4]. Inhibition of the cytolysis of uninfected cells is therefore considered to prevent or delay the onset of AIDS. On the other hand, it seems now established that the Nef protein of HIV-1 contributes substantially to pathogenesis of AIDS, although the mechanism remains unclear. We have shown that the Nef protein, found on the surface of virus-infected cells [5] and in a soluble form in sera of HIV-1-infected patients [6], binds to a broad spectrum of uninfected blood cells and, upon cross-linking by anti-Nef antibodies, it induces apoptotic cytolysis of the cells independently of CD95 (Fas) [6–8]. Cell surface expression of a putative Nef-binding protein, which occurs with various cell types much broader than those expressing Fas, depends on cellular activation [8]. On the other hand, various murine blood cells are also competent as a target of Nef-induced apoptosis in a similar fashion to human cells [9]. The Nef-induced apoptosis of murine cells is specifically inhibited by serine/threonine protein kinase (PK) inhibitors not inhibiting Fas-mediated apoptosis, suggesting that the Nef-induced apoptosis undergoes through a signal transduction pathway distinct from the Fas-mediated one in murine cells [9]. In this communication, we have examined inhibitory effects of the PK inhibitors on the Nef-induced and Fas-mediated apoptoses of uninfected human blood cells and discussed the feasibility of clinical use of the drugs to prevent the development of AIDS.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant soluble Nef proteins of the IIIB and ELI strains of HIV-1 were from Intracel, Cambridge, USA and Immuno Diagnostics, Inc., New York, USA, respectively. A serine/threonine PK inhibitor, fasudil hydrochloride (hexahydro-1-[5-isoquinolinesulfonyl]-1H-1,4-diazepine hydrochloride) and its metabolite M3 (1-[1-hydroxy-5-isoquinolinesulfonyl]-homopiperazine) [10] were from Asahi Chemical Industry Co. Ltd., Japan. H-7 (1-[5-isoquinolinylsulfonyl]-2-methylpiperazine) was from Sigma Chemical Co., USA. IL-1β converting enzyme (ICE) inhibitor I (acetyl-Try-Val-Ala-Asp-CHO) was from Calbiochem, La Jolla, USA. Mouse monoclonal antibodies (mAbs) to the N-terminus of Nef protein (BRU strain) and to human CD95 (Fas) (CH-11) were from Advanced Biotechnologies, Inc., Columbia, USA, and Medical and Biochemical Laboratories, Co., Ltd. Japan, respectively. Mouse mAbs against human CD25 and CD38 were from PharMingen, San Diego, USA.

2.2. Cells

Human CD4$^+$ T cell lines derived from lymphoma (H9), lymphoblastoma (CEM-5) and T cell leukemia (Jurkat); CD4$^+$ CD8$^+$ T cell line from lymphosarcoma (Tall-1); CD8$^+$ T cell line from normal peripheral blood (5B5); B cell line from Burkitt lymphoma (Ramos); monocyte line from histiocytic lymphoma (U937); and neutrophil lines from myelogenous leukemia (K562) and promyelocytic leukemia (HL60) were used as described previously [8]. Peripheral blood mononuclear cells (PBMC) from healthy adults were incubated for 2 to 4 days in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in the presence of 1 μg/ml phytohemagglutinin (PHA).

2.3. Assay of Nef protein bound to cell surfaces

Cell suspension was incubated with 1 μg/ml Nef protein for 40 min at 4°C. After washing with phosphate buffered saline (pH 7.2, PBS), cells were stained with anti-Nef mAb and FITC-labeled secondary Ab, and analyzed for the amount of Nef bound to the cell surfaces by flowcytometry [8]. To analyze the effect of PK inhibitors on the cell

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surface expression of a putative Nef-binding protein, PBMC were activated with 1 μg/ml PHA for 2 days at 37°C in the presence of various concentrations of PK inhibitors [9]. Cell line cells were likewise preincubated with the drugs for various periods. The cells were washed with PBS and subjected to the Nef-binding assay as above.

2.4. Analysis of apoptotic cytolysis induced by Nef protein and anti-CD95 (Fas) mAb

The procedure described previously [8] was used with slight modification. Briefly, cell suspension (10^5 cells in 100 μl RPMI-1640 medium with 10% FCS) was incubated either with 100 ng/100 μl Nef protein for 2 h on ice, anti-Nef mAb for 30 min on ice and then in a 96-well plate coated with anti-mouse IgG Ab for 2 days at 37°C, or with 1 μg/ml anti-human CD95 (Fas) mAb for 6 h at 37°C. The cells were treated with the lysis solution (0.1% Triton X-100 and 50 μg/ml propidium iodite in 0.1% sodium citrate) and the nuclear fraction was analyzed for DNA fragmentation by flowcytometry.

2.5. Inhibition of Nef-induced and Fas-mediated apoptoses by PK and ICE inhibitors

PBMC activated with PHA and cell line cells were preincubated with various concentrations of PK inhibitors or ICE inhibitor I for various periods at 37°C. The cells were then assayed for Nef-induced apoptosis and Fas-mediated apoptosis as described above in the presence of each drug.

3. Results

3.1. Inhibitory effect of ICE inhibitor on Nef-induced apoptosis of human blood cells

Since Fas-mediated apoptosis has been shown to be inhibited by ICE inhibitors, ICE inhibitor I was examined for inhibitory effect on the Nef-induced apoptosis. The ICE inhibitor suppressed Fas-mediated apoptosis of human blood cells caused by anti-Fas mAb, which occurred with restricted cell types expressing Fas (CEM-5, H9, Jurkat and PHA-activated PBMC), as reported previously [11] (Table 1). Apoptotic cytolysis induced by the Nef protein of HIV-1 (IIIB strain), which occurred with a broader spectrum of cell types than those for Fas-mediated apoptosis [8], was also inhibited by the ICE inhibitor (Fig. 1, Table 1). Similar results were obtained for the Nef of HIV-1 ELI strain (data not shown).

3.2. Inhibition of Nef-induced apoptosis of human blood cells by PK inhibitors

Since Nef-induced apoptosis of murine lymphoid cells is suppressed by serine/threonine PK inhibitors [9], we examined the effect of the drugs on the apoptosis of human blood cells. Whereas PK inhibitors, H-7, fasudil hydrochloride and its active form metabolite M3, did not inhibit the Fas-mediated apoptosis, these drugs strongly inhibited the Nef-induced apoptosis of all cell lines tested and PHA-activated PBMC (Fig. 1, Table 1). Essentially identical results were obtained for the Nef protein of IIIB and ELI strains of HIV-1. The PK inhibitors did not affect the Nef-binding capacity of the cell line cells and activated PBMC. Treatment of the soluble Nef protein with the drugs affected neither the cell surface-binding activity nor the apoptosis-inducing activity of Nef (data not shown). Fasudil hydrochloride and M3 inhibited the Nef-induced apoptosis in a dose-dependent manner, in parallel with the inhibitory effect on PKC enzyme activity in vitro [12], showing a complete inhibition at 1 to 10 μM (Fig. 2) and no cytotoxicity at least at 30 μM for all cell types tested (data not shown). On the other hand, H-7 suppressed cell proliferation to some extent at 1 μM, a concentration inhibiting the Nef-induced apoptosis.

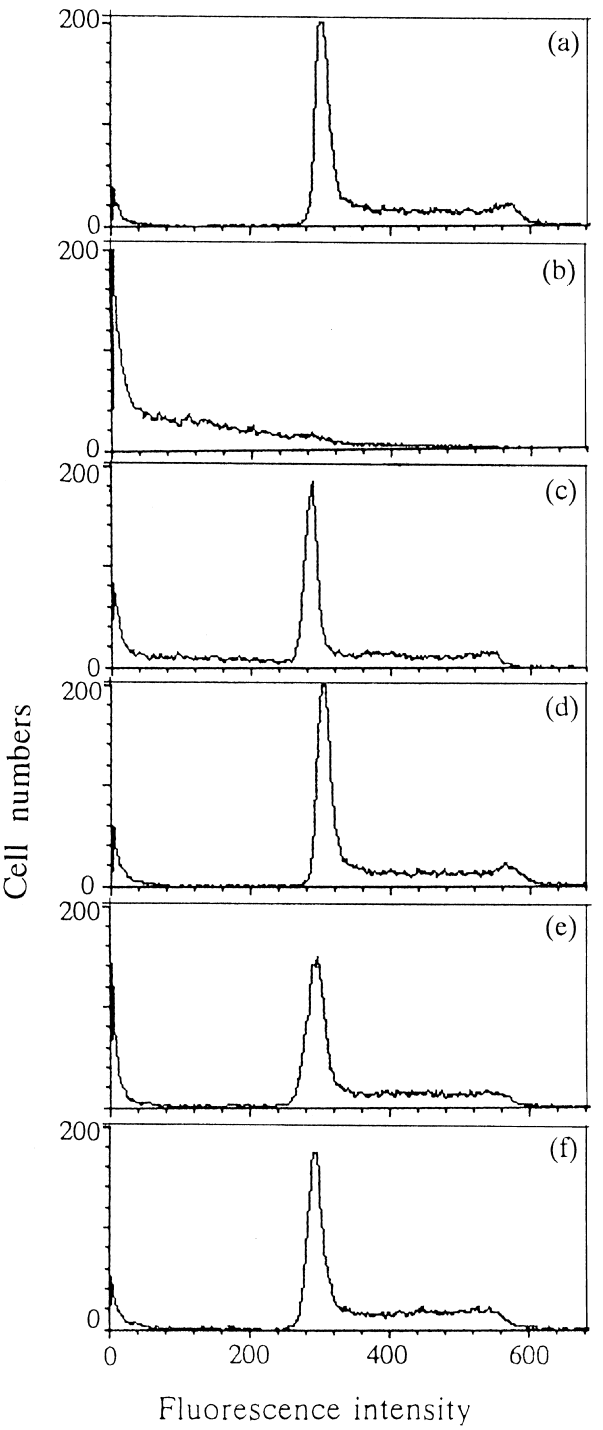

Fig. 1. Effect of inhibitors on the Nef-induced apoptotic cytolysis of Jurkat cells. Cell suspension (10^5 cells in 100 μl RPMI-1640 medium with 10% FCS) was pretreated with inhibitors for 30 min at 37°C, then incubated with HIV-1 Nef protein (IIIB strain, 100 ng/100 μl) in the presence of drugs for 48 h at 37°C, and analyzed for DNA fragmentation by flowcytometry. a: Control, without Nef and drugs. b: Nef without drugs. c: Nef and 1 μM fasudil hydrochloride. d: Nef and 1 μM M3. e: Nef and 1 μM H-7. f: Nef and 1 mM ICE inhibitor I.

Pretreatment of target cells with the PK inhibitors before incubation with the Nef protein in the presence of each inhibitor was required for the inhibition of Nef-induced apoptosis.

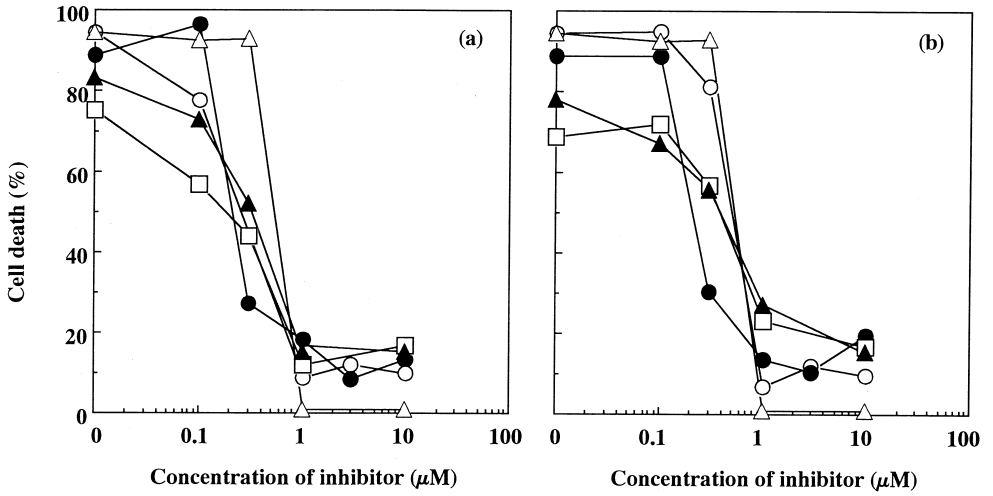

Fig. 2. Dose-dependent inhibitory effects of fasudil hydrochloride (a) and M3 (b) on the Nef-induced apoptotic cytolysis of cell line cells. The procedure described in the legend for Fig. 1 was followed using PK inhibitors at indicated concentrations. (○) H9, (△) Jurkat, (□) Ramos, (●) U937, and (▲) K562 cells.

The inhibitory effect depended on both the preincubation period and concentration of the drugs. Preincubation of cell line cells for more than 30 min and of PBMC for more than 60 min, respectively, was needed for efficient inhibition by M3 at 1 μM, while a higher concentration and a longer preincubation period were required for fasudil hydrochloride (Fig. 3).

3.3. *Inhibition of cell surface expression of a putative Nef receptor in PBMC by PK inhibitors*

The Nef protein, at the C-terminal domain, binds to cell surfaces of various human blood cell line cells, which are more or less activated [6–8]. Although Nef does not bind to PBMC in a resting stage, stimulation with PHA confers the Nef-binding capacity on a part of the cells [8]. These findings suggest the presence of a putative Nef-binding protein (receptor) responsible for the Nef-induced apoptosis and that cell surface expression of the receptor molecule depends on cellular activation [6–8]. Although the PK inhibitors did not interfere with the Nef binding to activated cells as described above, a possibility remained that the drugs suppressed cell surface expression of the putative Nef receptor. Incubation of cell line cells with the PK inhibitors for 2 days affected neither the Nef-binding capacity nor cell surface markers of cellular activation, CD25 and CD38 (data not shown). On the other hand, cell surface expression of the Nef-binding capacity of PBMC, which was induced by incubation of the resting cells with PHA for 2 days, was completely inhibited by fasudil hydrochloride and M3 at 10 μM (Fig. 4) and thereby, the PBMC remained insusceptible to the Nef-binding and Nef-induced apoptosis. However, the treatment with the PK inhibitors did not influence the expression of CD25 and CD38, cell surface markers of cellular activation (data not shown).

4. Discussion

Extensive destruction of a variety of uninfected blood cells

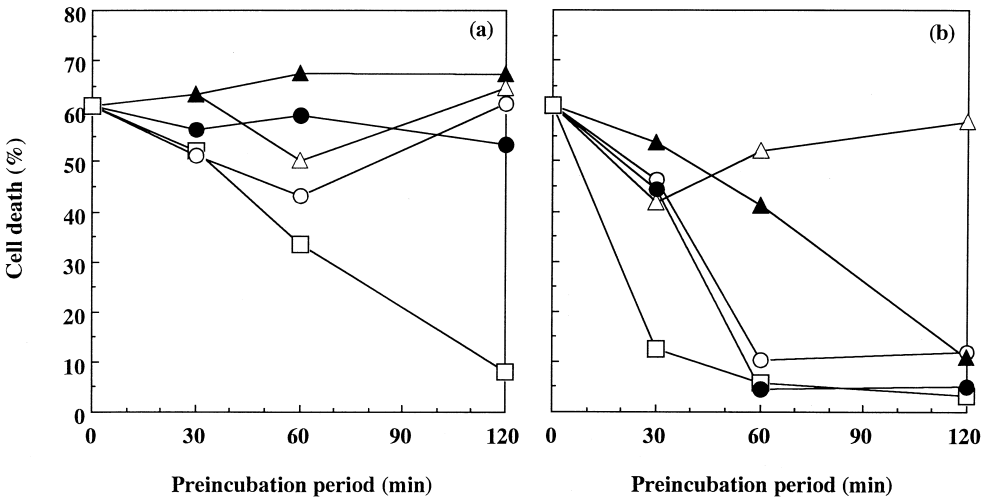

Fig. 3. Effects of preincubation period and concentration of PK inhibitors on inhibition of the Nef-induced apoptosis of PBMC. PBMC from healthy adult were activated with 1 μg/ml PHA for 2 days. The cells were then subcultured with various concentrations of fasudil hydrochloride (a) and M3 (b) for indicated periods before subjected to the assay of Nef-induced apoptosis in the presence of each drug as described in the legend for Fig. 1. Concentrations of the PK inhibitors were 0.1 μM (△), 0.3 μM (▲), 1 μM (○), 3 μM (●), and 10 μM (□).

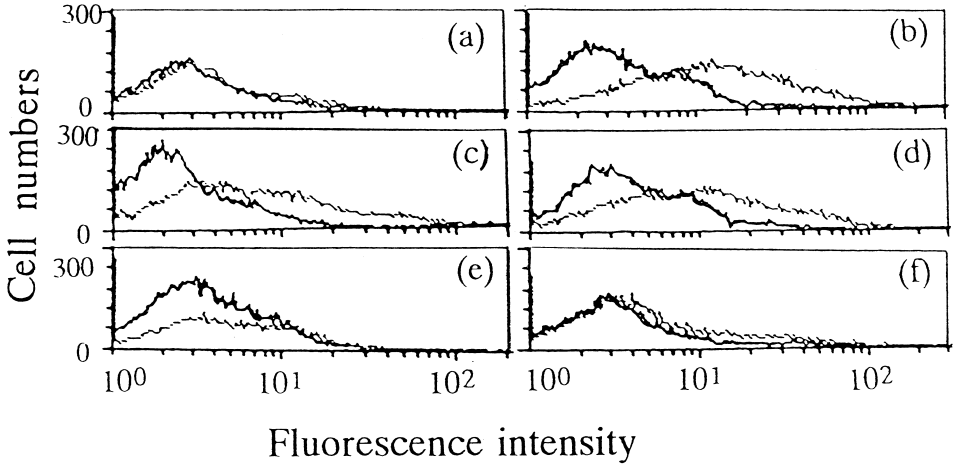

Fig. 4. Inhibitory effect of PK inhibitors on cell surface expression of Nef-binding capacity of PBMC. PBMC were activated with 1 μg/ml PHA for 2 days in the presence of PK inhibitors. The cells were then treated with 1 μg/ml Nef protein (IIIB strain) for 40 min at 4°C, stained with anti-Nef mAb and FITC-conjugated secondary Ab and assayed for the amount of Nef by flowcytometry. Bold lines, without anti-Nef mAb; thin lines, with anti-Nef mAb. Control cells before PHA treatment (a). PHA-activated cells in the presence of no inhibitor (b), 1 μM M3 (c), 10 μM M3 (e), 1 μM fasudil hydrochloride (d) or 10 μM fasudil hydrochloride (f).

during the subclinical stage of HIV-1 infection is likely responsible for the development of AIDS [1–4]. Tat and gp120 of HIV-1, which induce Fas-dependent apoptosis of restricted cell types [13], may contribute in part to the depletion of uninfected cells. On the other hand, we have postulated the involvement of Nef-induced apoptosis in this event [6–8] and searched for drugs inhibiting the cell death [9]. The present study demonstrated that the Nef-induced apoptotic cytolysis of a broad spectrum of human blood cells was efficiently inhibited by serine/threonine PK inhibitors, H-7, fasudil hydrochloride and its metabolite M3, which did not inhibit Fas-mediated apoptosis. These results appeared quite similar to those obtained for murine lymphoid cells [9]. The Nef-induced apoptosis, occurring with various cell types irrespective of the presence of Fas [8], was also inhibited by ICE inhibitor I, which affects the lower stream of the Fas-mediated apoptotic pathway [11]. These results suggest that although the upper stream of signal transduction pathway of the Nef-induced apoptosis is different from that of the Fas-mediated one, they meet each other upstream of ICE into a common track leading to apoptosis. Since the PK inhibitors did not affect the binding of Nef to cell surfaces, the drugs likely inhibited phosphorylation of cellular protein(s) involved in the affluent stream of the Nef-specific pathway.

Pretreatment of the cells with the PK inhibitors was required for the inhibition, suggesting that once after the apoptosis-inducing signal is transduced by phosphorylation of the protein(s), the PK inhibitors can no longer block the signal. A longer preincubation period for fasudil hydrochloride was required than for M3, probably because fasudil hydrochloride taken up by cells was converted to an active form M3 during this period [10], and thereby impeded transduction of the apoptosis-inducing signal introduced by binding of Nef to a putative Nef receptor, a death-signalling receptor. The present results strongly suggest that the Nef protein serves as a ligand to such a putative Nef receptor, which may represent a member of the tumor necrosis factor (TNF) receptor family [14]. Considering biological nature and spectrum of the Nef-induced apoptosis [6–9], TNF-related apoptosis-inducing ligand (TRAIL, Apo-2L) receptor [15–17] could be a most likely candidate. Molecular characterization of the Nef-induced apoptotic pathway is in progress.

Table 1
Inhibitory effects of drugs on Nef-induced and Fas-mediated apoptoses

Cells	Nature	Nef-induced apoptosis[a] (%)					Fas-mediated apoptosis[b] (%)				
		No drug	H-7 (1 μM)	Fasudil (1 μM)	M3 (1 μM)	ICE inhibitor I (1 mM)	No drug	H-7 (1 μM)	Fasudil (1 μM)	M3 (1 μM)	ICE inhibitor I (1 mM)
CEM-5	$CD4^+$ T	93	6	18	16	23	22	27	31	21	1
H9	$CD4^+$ T	92	9	11	7	7	86	91	79	83	7
Jurkat	$CD4^+$ T	87	4	1	4	1	70	73	72	67	1
Tall-1	$CD4^+$ $CD8^+$ T	74	4	9	1	2	1	–	–	–	–
5B5	$CD8^+$ T	73	13	17	18	15	23	n.t.[c]	n.t.	n.t.	n.t.
Ramos	Blymphocyte	92	6	12	23	21	3	–	–	–	–
K562	Neutrophil	77	8	17	27	30	1	–	–	–	–
HL60	Neutrophil	83	9	23	21	17	3	–	–	–	–
U937	Macrophage	95	11	18	19	30	8	–	–	–	–
PBMC	Normal	<5	–	–	–	–	2	–	–	–	–
PBMC	PHA activated[d]	64	7	8	7	7	37	40	35	37	7

[a]Cells (10^5 cells/100 μl) were incubated with 100 ng Nef (IIIB strain) and anti-Nef mAb for 48 h at 37°C.
[b]Cells (10^5 cells/100 μl) were incubated with 100 ng mAb CH-11 for 6 h at 37°C.
[c]Not tested.
[d]Cells were activated with 1 μg/ml PHA for 2 or 4 days for assay of Nef-induced or Fas-mediated apoptosis, respectively.

In addition, treatment of PBMC, and not cell line cells, with the PK inhibitors suppressed cell surface expression of the Nef-binding capacity, which is associated with cellular activation and is a prerequisite for the Nef-induced apoptosis [8], without affecting cell surface markers of cellular activation. These results suggest that cell surface expression of the putative Nef receptor also depends on phosphorylation of a cellular protein which is specifically inhibited by the PK inhibitors. Cell line cells, on the other hand, may have escaped such regulatory mechanism. PBMC of HIV-1-infected patients would be activated by cytokines induced by HIV-1 and other opportunistic infections. Therefore, selective inhibition of the cell surface expression of the Nef receptor by the PK inhibitors might also prevent the Nef-induced cytolysis of activated cells.

Fasudil hydrochloride and M3 specifically and efficiently inhibited the Nef-induced apoptotic cytolysis, which occurs with a broader spectrum of cell types than those for Fas-mediated apoptosis [8], and showed no cytotoxicity. Fasudil hydrochloride has already been used as a therapeutic of subarachnoidal hemorrhage to inhibit contraction of blood vessels in the brain [18]. The present results taken together suggest the feasibility of clinical use of the PK inhibitors to prevent or delay the development of AIDS by inhibiting the Nef-induced cytolysis of a broad spectrum of uninfected and activated blood cells, in addition to suppressing TNF-induced replication of the HIV-1 genome in infected cells [19].

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