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Effects of SKF 108922, an HIV-1 protease inhibitor, on retrovirus replication in mice

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Abstract

Rationally designed synthetic inhibitors of retroviral proteases inhibit the processing of viral polypeptides in cultures of human T lymphocytes infected with human immunodeficiency virus type 1 (HIV-1) and therefore suppress the infectivity of HIV-1 in vitro. We have previously reported the antiviral activity in vitro of HIV-1 protease inhibitors against the C-type retrovirus Rauscher murine leukemia virus (RMuLV) and the lentivirus simian immunodeficiency virus (SIV). The same compounds which blocked the infectivity of HIV-1 also inhibited the infectivity of RMuLV and SIV in vitro. This report extends these findings by testing the antiviral activity of HIV-1 protease inhibitors in vivo in the RMuLV model. RMuLV-infected mice were treated twice a day (bid) with either an active (SKF 108922) or inactive (SKF 109273) compound for fourteen days by the intraperitoneal (IP) route. Compared with excipient control, SKF 108922, formulated with hydroxypropyl-β-cyclodextrin (HPB), reduced virus-induced splenomegaly, viremia, and serum reverse transcriptase (RT) levels, while SKF 109273 was inactive. The HPB vehicle by itself enhanced replication of RMuLV. The effects of changing the formulation and the route of administration were examined. SKF 108922, formulated in HPB, had similar antiviral activity when administered by the IP or subcutaneous (SC) routes. However, SKF 108922 administered as a colloidal suspension in cholesterol sulfate (CS) had no detectable antiviral effect. Measurements of the circulating levels of the protease inhibitor in plasma explained this result. Plasma concentrations of SKF 108922 exceeded 1000 nM within 10 min after SC administration of the compound solubilized in HPB, but SKF 108922 was not detected in plasma after SC administration of the same dose formulated with CS. Information on optimal conditions for administering these agents should prove useful in guiding their clinical application Therefore, RMuLV should provide a good model for the preclinical evaluation and development of this class of agents for the treatment of HIV.

Keywords: Protease inhibitor; Animal model; Murine retrovirus; Murine leukemia virus

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1. Introduction

The maturation of retroviruses requires the action of a virally encoded protease (Crawford and Goff, 1985; Katoh et al., 1985; Kohl et al., 1988; Seelmeier et al., 1988; Gottlinger et al., 1989; Navia et al., 1989). The mRNA of retroviruses is translated into polyproteins which must be cleaved by the viral protease before the immature virus particles can acquire infectivity. Studies on inhibition of the function of retroviral proteases by mutation (Katoh et al., 1985; Kramer et al., 1986; Kohl et al., 1988; Mous et al., 1988; Seelmeier et al., 1988; Gottlinger et al., 1989; Peng et al., 1989) or by specific synthetic inhibitors (Meek et al., 1990; McQuade et al., 1990; Roberts et al., 1990; Lambert et al., 1992; Black et al., 1993) have established the central role of these viral enzymes in viral maturation.

Retroviral proteases belong to the class of aspartic proteases, all of which contain a pair of highly conserved regions composed of two hydrophobic amino acids followed by the characteristic sequence Asp-Thr-Gly (Katoh et al., 1987; Kräusslich and Wimmer, 1988; Meek et al., 1989). Furthermore, retroviral proteases form homodimers in the active state (Pearl and Taylor, 1987a; Meek et al., 1989; Miller et al., 1989; Navia et al., 1989; Wlodawer et al., 1989). The overall conservation in tertiary and quaternary structure, especially in the region of the active site of retroviral proteases, leads to similarities in the cleavage site sequences which they recognize (Pearl and Taylor, 1987b; Kräusslich and Wimmer, 1988). These similarities suggest that synthetic protease inhibitors with demonstrated activity against HIV-1 could inhibit the infectivities of animal retroviruses. Animal retrovirus models could then serve as a guide for the preclinical development of protease inhibitors for AIDS therapy.

In fact, we have previously reported that HIV-1 protease inhibitors effectively inhibit replication of murine retroviruses in vitro (Black et al., 1993). In those and the current studies, the inhibitors are peptide substrate analogs in which the scissile dipeptide linkage is substituted with a non-scissile hydroxyethylene isostere (Dreyer et al., 1992). We examined the antiviral activities of six protease inhibitors against HIV-1, Rauscher murine leukemia virus (RMuLV), and simian immunodeficiency virus (SIV). We found that compounds, designated SKF 108390, 108842, and 108922 had similar levels of antiviral activity against all three retroviruses, with IC_{50} values and ≤ 1 μM (Black et al., 1993).

Although RMuLV, a C-type retrovirus, has a completely different disease pathology in the mouse than HIV, a lentivirus, has in the human, it is a useful in vivo model for evaluating the effects of an antiviral agent on virus infection and replication. This model should predict whether in vivo efficacy with an antiviral compound is feasible. Consequently, the ability of an HIV-1 protease inhibitor, which is active against both RMuLV and HIV-1, to reduce the levels of infectious RMuLV titers in the serum of mice is perhaps the most important predictor of a protease inhibitor's antiviral action.

Understanding the mechanism of pathogenesis of RMuLV (Rauscher, 1962; Chirigos, 1964; Gardner and Luciw, 1989; Ruprecht et al., 1990) is important for the correct interpretation of how a protease inhibitor might work in this model. In the RMuLV model, the pathology is generated by the interaction of two different retroviruses in the virus complex: (1) a replication-competent helper virus, which induces B-cell lymphomas late in the life of infected animals, giving rise to Rauscher murine leukemia in mice; and (2) a replication-defective Rauscher Spleen Focus Forming Virus (RSFFV), which induces a rapidly fatal erythroid disease (Ruprecht et al., 1990). The initial rapid erythroid expansion is followed by the development of frank erythroleukemia. The major advantage of the RMuLV model is the susceptibility of adult animals to viremia and disease. Eight days after inoculation with virus, erythroid colonies are formed in the spleen, with the number of colonies being proportional to the virus titer. Each spleen colony presumably represents a successful viral hit, suggesting that the disease is polyclonal (Ruprecht et al., 1990). Continued viremia leads to palpable splenomegaly at 14 days post inoculation. By 21 days after inoculation, the spleens are enlarged massively, and animals succumb to erythroleukemia typically within four to six weeks

after infection. The degree of splenomegaly measured on day 20 after infection is proportional to the viral titer in the serum (Chirigos, 1964; Gardner and Luciw, 1989; Ruprecht et al., 1990).

The replication-defective component SFFV encodes a characteristic *env* product, gp54, which is necessary for the transforming properties of the virus (Berger et al., 1985; Bestwick et al., 1985; Chung et al., 1987; Wolff and Ruscetti, 1988). gp54 is a modified *env* gene product derived by a series of events including: (1) acquisition of an altered sequence by recombination with an endogenous provirus *env* gene; (2) a deletion extending into the TM (transmembrane) coding region; and (3) a rearrangement extending into the membrane-spanning domain (Coffin, 1985). The rapid overgrowth of erythroblasts in the mouse can be induced, albeit with reduced efficiency, by nondefective virus alone, but the defective component accelerates the process and gives rise to rapid focal proliferation of: (1) erythroblasts in spleens of infected mice; and (2) colonies of erythroid cells after infection of bone marrow cell cultures in vitro. Indeed, the gp54 oncogene alone can induce erythroblastosis (Wolff and Ruscetti, 1988).

In this communication we extend our previous report of the in vitro activity of HIV-1 protease inhibitors against RMuLV (Black et al., 1993). We report here the antiviral activity in RMuLV-infected mice of SKF 108922, a synthetic peptide analog inhibitor of HIV-1 protease.

2. Materials and methods

2.1. Protease inhibitor

The structure of SKF 108922 is Cbz-Ala-Phe Ψ[(*S*)- CH(OH)CH_2]Ala-Val-Valinol, where Phe Ψ[(*S*)-CH(OH)CH_2]Ala is the hydroxyethylene isostere of Phe-Ala and Cbz is benzyloxycarbonyl. SKF 108922 was synthesized by methods similar to those previously described (Dreyer et al., 1989, 1992). Briefly, the synthesis proceeded as follows: mixed anhydride coupling of Phe Ψ[(*S*)-CH(OSiMe$_2$*t*Bu)CH_2]Ala to valinol (using iBuO-COCl and *N*-methyl morpholine); selective removal of the Boc group with trifluoroacetic anhydride; mixed anhydride coupling to Cbz-Ala; and finally, removal of the SiMe$_2$*t*Bu group with trifluoroacetic anhydride. The inhibitor was fully characterized by ^{1}H-NMR, mass spectrometry, and combustion analysis. SKF 108922 exhibited an apparent inhibition constant (K_i) of 2 nM with recombinant HIV-1 protease when assayed as previously described (Dreyer et al., 1992). In contrast, SKF 109273 displayed no antiviral activity against RMuLV in vitro (Black et al., 1993), and it was used as a control in some experiments. SKF 109273, which was also designated SKF 109274 in our previous publication (Black et al., 1993), has the structure Boc-Phe and Ψ[$COCH_2$]Gly-Val-Val-OCH_3, where Boc is *tert*-butyloxycarbonyl. AZT was purchased from Sigma (St. Louis, MO).

2.2. Virus stocks

The RMuLV stock was originally obtained from Dr. Ruth Ruprecht (Children's Medical Center, Boston, MA). Virus stocks were subsequently prepared by passage in vivo in BALB/c mice inoculated intraperitoneally (IP) with virus. Two or three weeks after infection, the mice were anesthetized and exsanguinated by cardiac puncture, and spleens were removed and weighed. Splenic homogenates or serum from infected animals were used for subsequent infections. Virus stocks were titrated both in vivo and in vitro in SC-1 cells.

2.3. Virus titration assays

Serum samples were used to determine reverse transcriptase (RT) levels and viral titers. We measured serum RT with a micro assay (Chirigos et al., 1990), using nucleotide labelled with ^{32}P or ^{3}H, similar to the one used for measuring HIV RT (Goff et al., 1981; Meek et al., 1990; Lambert et al., 1992), except that Mn was substituted for Mg due to the different divalent cation requirements of the MuLV protease. Infectious RMuLV titers were also determined by a UV-XC assay (Rowe et al., 1970; Chirigos et al., 1990) or a p30 ELISA endpoint dilution method (Hollingshead et al., 1992) to determine the 50% tissue culture infec-

tious dose ($TCID_{50}$). In addition, total spleen cell counts, and differential cell counts were obtained for each animal. Spleen cell lysates were also analyzed by SDS-PAGE and Western blot for processing inhibition of virion gag proteins (Black et al., 1993).

2.4. In vitro compound testing

Anti-RMuLV activity of SKF 108922 was confirmed in vitro using the SC-1 cell line and the RMuLV p30 enhanced chemiluminescence plaque assay (Barney et al., in preparation) before consideration for in vivo experiments.

2.5. Animals

Female BALB/c AN Ncr IBR inbred mice weighing from 16 to 18 grams were obtained from Charles River Laboratories (Raleigh, NC). Animals were housed ten per cage and allowed an acclimation period of five to seven days before use. Mice were randomized into groups and weighed on days −1 and 14.

2.6. In vivo experimental protocols

Mice (10/group) were treated with antiviral compounds by various routes including IP, subcutaneous (SC), and oral (in drinking water). Sterile, physiological saline, administered in the same volume and by the same schedule and route as the antiviral compounds being tested, served as the negative control. AZT (0.3 mg/ml, equal to approximately 50–75 mg/kg/day) was given orally in continuously available drinking water as a positive control for every experiment and resulted in 60–80% reduction in splenomegaly compared with infected control mice. This result is consistent with our extensive experience (and >20 experiments) with AZT in this model (Black et al., 1990) and with published results from other laboratories (Ruprecht et al., 1990). SKF 108922 and SKF 109273 were formulated with hydroxypropyl-β-cyclodextrin (HPB, Molecusol, Pharmatech, Alachua, FL) as a solution at 1.7 mg/ml final concentration for SC or IP administration (0.1–0.2 ml/dose). SKF 108922 was also formulated as a stabilized colloidal dispersion at 3 mg/ml with cholesterol-3-sulfate (Sigma) with a mean particle diameter of 2.1 μm by laser-light scattering. Cholesterol-3-sulfate has also been used in a novel formulation for the preparation of a colloidal dispersion of amphotericin B (Guo et al., 1991), and formulation screening studies in our laboratories suggested that a similar approach would be useful in formulating SKF 108922. SKF 108922 was tested for inhibition of RMuLV by administering different concentrations of compound formulated in HPB or cholesterol-3-sulfate either IP or SC twice per day (bid), and the dose of HPB or cholesterol-3-sulfate was kept constant in all administrations. Treatments began on day −1 and continued through day 13. On day 14, mice were anesthetized, weighed, and exsanguinated by cardiac puncture; and spleens were removed, weighed, and prepared for additional assays. Sera were collected and stored frozen at −70°C until used to determine viral titers and RT activity.

2.7. In vitro assays for in vivo efficacy

Spleen weights for control and test animals were determined immediately after sacrifice. Serum samples were used to determine RT levels and viral titers ($TCID_{50}$). The ^{3}H or ^{32}P based micro-RT assay was used (Chirigos et al., 1990; Lambert et al., 1992) with the substitution of Mn^{2+} for Mg^{2+}, and infectious RMuLV titers were determined using an XC assay or a p30 endpoint dilution method.

2.8. Measurement of circulating drug levels

In parallel experiments, SKF 108922 was administered SC to mice at a dose of 5 mg/kg in a volume of 0.1 ml, and blood samples were taken by cardiac puncture at 0, 10, 30, 60, 90, 120, 150, 180, 210, 270, 300, 330, 360, 390, 420, 480, 540, 600, 660, and 720 min after treatment. The blood was added to 0.1 M citrate buffer and spun down. Plasma samples were snap-frozen, and plasma drug concentrations were then measured by inhibition of purified HIV-1 protease, using a synthetic substrate (Dreyer et al., 1992; Black et al.,

1993; Abdel-Meguid et al., 1994). Briefly, standard curves were generated in triplicate from stock solutions spiked into mouse plasma. The spiked plasma solutions were extracted with acetonitrile, and the supernatant was taken to dryness by speed-vac. The resulting residue was assayed for HIV-1 protease inhibition by adding the protein substrate and then the HIV-1 protease. Initial rates were determined for each standard curve as the fraction of remaining enzymatic activity at each inhibitor concentration.

3. Results

3.1. Effect of vehicle on RMULV disease

Due to the insolubility of SKF 108922 and the other protease inhibitors in aqueous solution (< 0.03 mg/ml), it was necessary to formulate them in HPB in order to administer them to mice. As shown in Fig. 1, this vehicle by itself dramatically enhanced virus replication, as indicated by increases in splenomegaly, viremia, and serum RT levels. This enhancing effect was seen consistently in subsequent experiments, and therefore the RMuLV-infected and excipient-treated animals were used as the control group to compare the effects of protease inhibitors. Interestingly, AZT treatment completely overcame the virus-enhancing effect of HPB, and the results from animals treated with both HPB and AZT were indistinguishable from those from animals treated with AZT alone (data not shown).

3.2. Effect of protease inhibitors on RMULV disease

As shown in Fig. 1, the protease inhibitor SKF 108922 reduced splenomegaly, serum virus titer, and serum RT levels at all doses tested (11, 4.4, and 2.1 mg/kg, bid), compared with the vehicle control (the reduction in splenomegaly at 2.1 mg/kg was not statistically significant at the $P < 0.05$ level). The magnitude of the reductions in splenomegaly ranged from 35% to 48%; for viremia, from 71% to 81%; and for serum RT, from 60% to 70%. In contrast, SKF 109273, which had no antiviral activity in vitro against either HIV or RMuLV, did not protect mice against RMuLV disease. In addition, spleens, livers, and femoral bone marrow were collected from these mice, and the tissues were preserved in buffered formalin. Tissues were embedded, sectioned, stained with hematoxylin-eosin, and evaluated microscopically for evidence of leukemia, using a scoring scale of 0 (= no erythroleukemia) to 5 (= severe leukemia). The results of this evaluation (Table 1) were entirely consistent with the other measures of antiviral activity shown in Fig. 1. SKF 108922 reduced erythroleukemia, while SKF 109273 did not.

When this experiment was repeated with a wider range of doses of SKF 108922, a dose-dependent reduction of splenomegaly was seen: 49% at 68 mg/kg, 8% at 34 mg/kg, none at lower doses, (Fig. 2A), but these reductions were not statistically significant due to high variability in the data. Treatment of infected mice with SKF 108922 reduced the titers of infectious virus in serum by $\geq 90\%$ at doses ≥ 11 mg/kg. These differences were statistically significant at the $P < 0.05$ level, and the magnitude of these reductions was comparable to that of the AZT control (Fig. 2B).

3.3. Effects of route of administration and vehicle on antiviralactivity of SKF 108922

In addition, the effects of changing the formulation and the route of administration were examined in the experiment shown in Fig. 2. A dose of 11 mg/kg of SKF 108922, formulated in HPB, given by the SC route had a similar effect as the same dose given by the IP route. This dose of SKF 108922 did not significantly affect splenomegaly by either route (Fig. 2A), but did dramatically reduce viremia by $> 80\%$, by both routes of administration (Fig. 2B). In contrast, a higher dose of SKF 108922 (26 mg/kg) administered as a colloidal dispersion in cholesterol sulfate by the SC route had no significant antiviral effect, as measured by splenomegaly or viremia (Fig. 2). As discussed above, a similar dose (34 mg/kg) of SKF 108922 solubilized in HPB and administered by the IP route significantly reduced viremia.

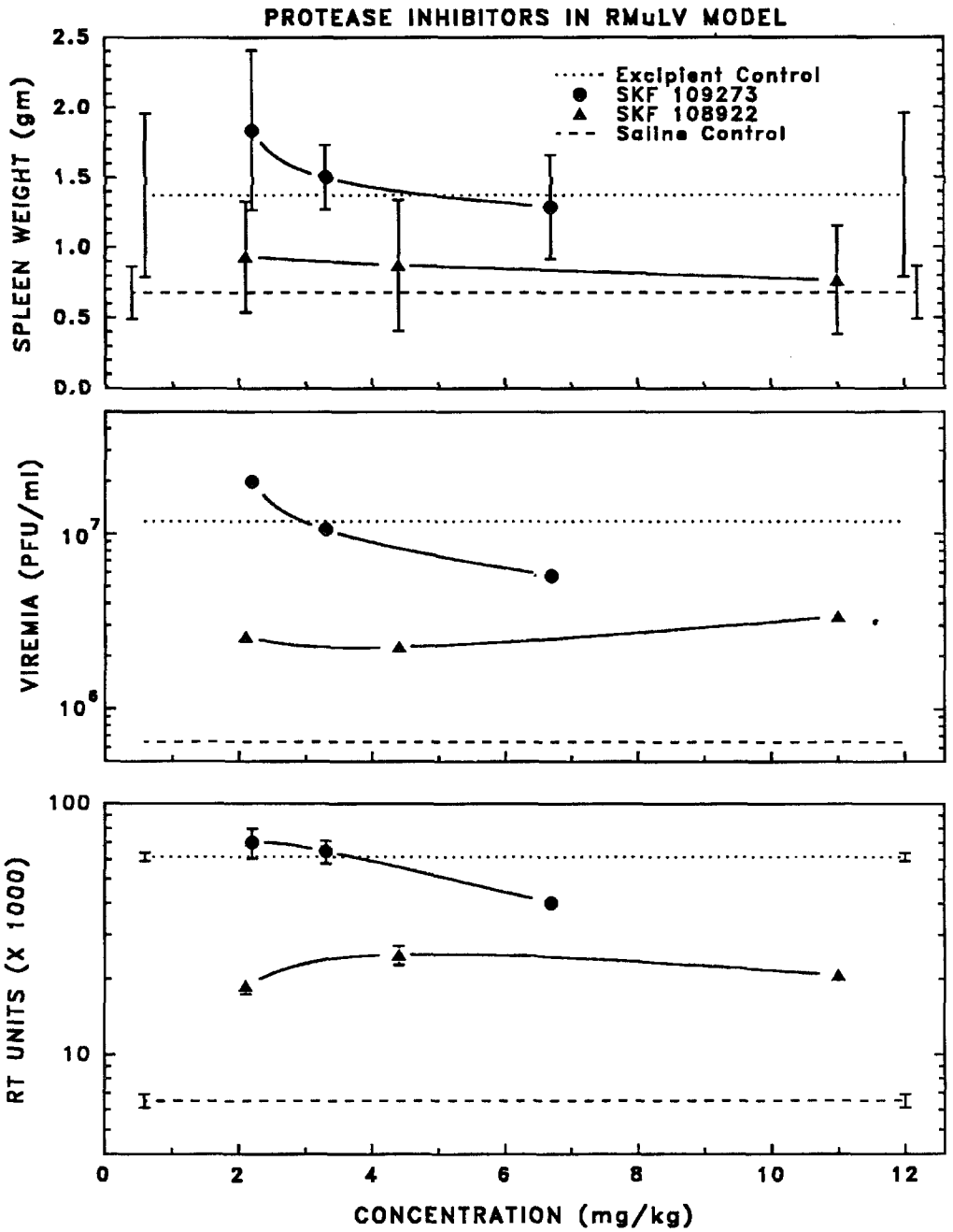

Fig. 1. Effect of protease inhibitors on splenomegaly (top panel), viremia (middle panel), and serum RT levels (bottom panel) in RMuLV model. Mice were inoculated IP with RMuLV on day 0, and treatment was begun on day and − 1 and continued through day 13. Protease inhibitors, formulated with HPB, were administered IP, bid, in a volume of 0.1 and –;0.2 ml/dose. The doses indicated on the abscissa are the total daily doses of protease inhibitors, while the dose of HPB was kept constant in all administrations. Sterile, isotonic saline was administered under similar conditions to the saline control (infected) group. Mice were euthanized on day 14. Viremia was measured in sera by UV-XC assay, with the results expressed as plaque-forming units (PFU)/ml. Serum RT was measured in a ^{3}H-based micro assay, with a unit of RT activity defined as the number of picomoles of [^{3}H]-dTTP substrate incorporated in 1 h by 1 ml of serum. The statistical significance of the differences in spleen weight was tested by analysis of variance (ANOVA). *P* Values were 0.021, 0.049, and 0.075 for the 11, 4.4, and 2.1 mg/kg/day doses, respectively.

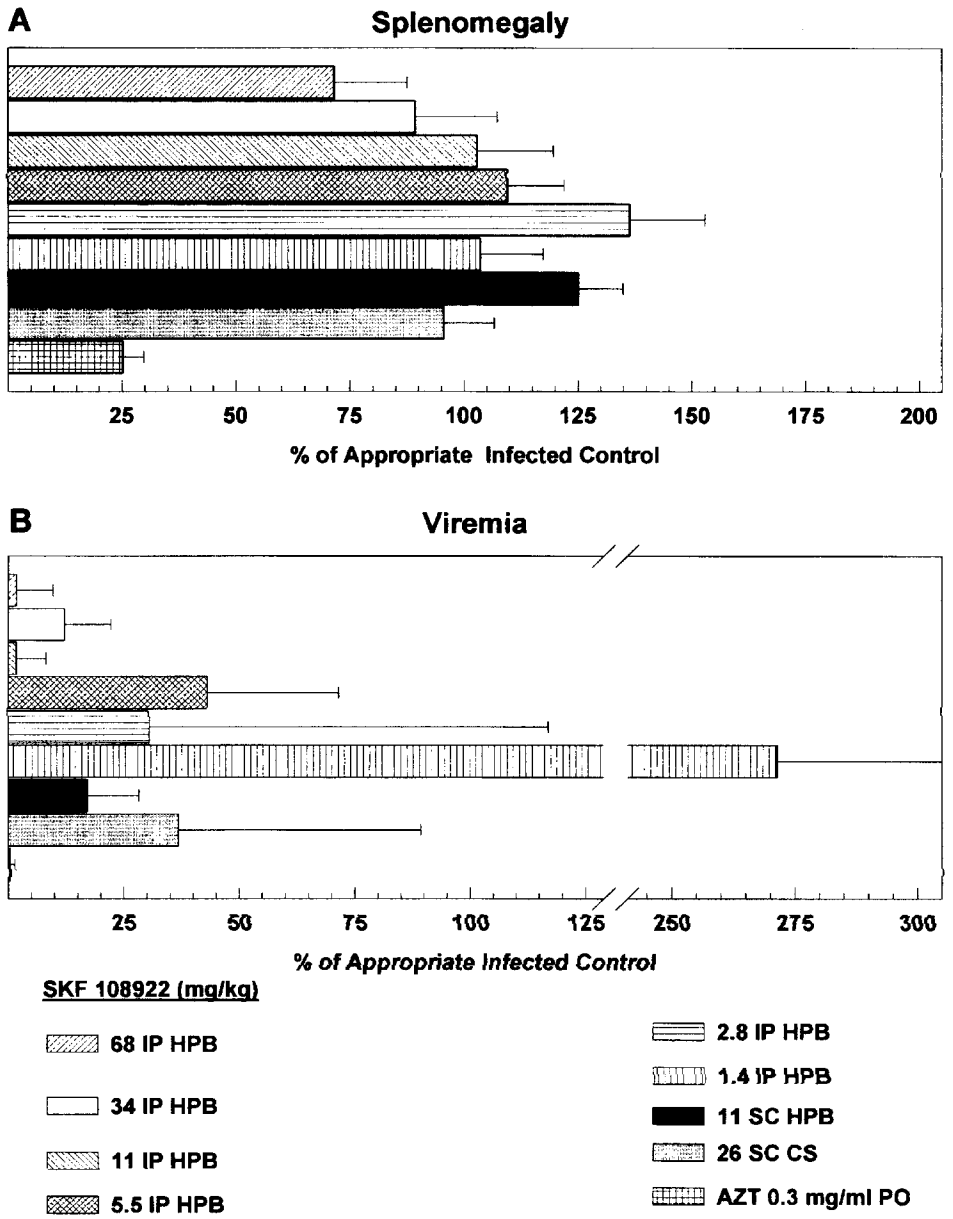

Fig. 2. Effect of protease inhibitors on splenomegaly (panel A) and viremia (panel B) in RMuLV model. Conditions were as described in Fig. 1, with exceptions noted. AZT (0.3 mg/ml, equal to approximately 50–75 mg/kg/day) was given orally in continuously available drinking water. Viremia was measured in sera by p30 ELISA. Results are expressed as % of the appropriate vehicle control, HPB or cholesterol sulfate (CS), and geometric mean and standard error are shown. Values of spleen weight for untreated, infected controls were: saline, 0.35 and $\pm$;0.038; HPB-IP, 0.71 and $\pm$;0.13; HPB-SC, 0.72 and $\pm$;0.12; CS-SC, 0.57 and $\pm$;0.052. Virus titers for untreated, infected controls were: saline, $3.1 \times 10^3 \pm 1.4 \times 10^3$; HPB-IP, $5.9 \times 10^4 \pm 1.4 \times 10^5$; HPB-SC, $1.1 \times 10^5 \pm 1.1 \times 10^5$; CS-SC, $1.4 \times 10^4 \pm 1.2 \times 10^4$. The data were subjected to ANOVA, and for spleen weight, all P values exceeded 0.05, except for AZT ($P < 0.001$). For viremia, the statistically significant P values were: SKF 108922 at 68 mg/kg/day, $P = 0.01$; at 34 mg/kg, $P = 0.033$; at 11 mg/kg (IP in HPB), $P = 0.009$; at 11 mg/kg (SC in HPB), $P = 0.032$; AZT, $P = 0.001$.

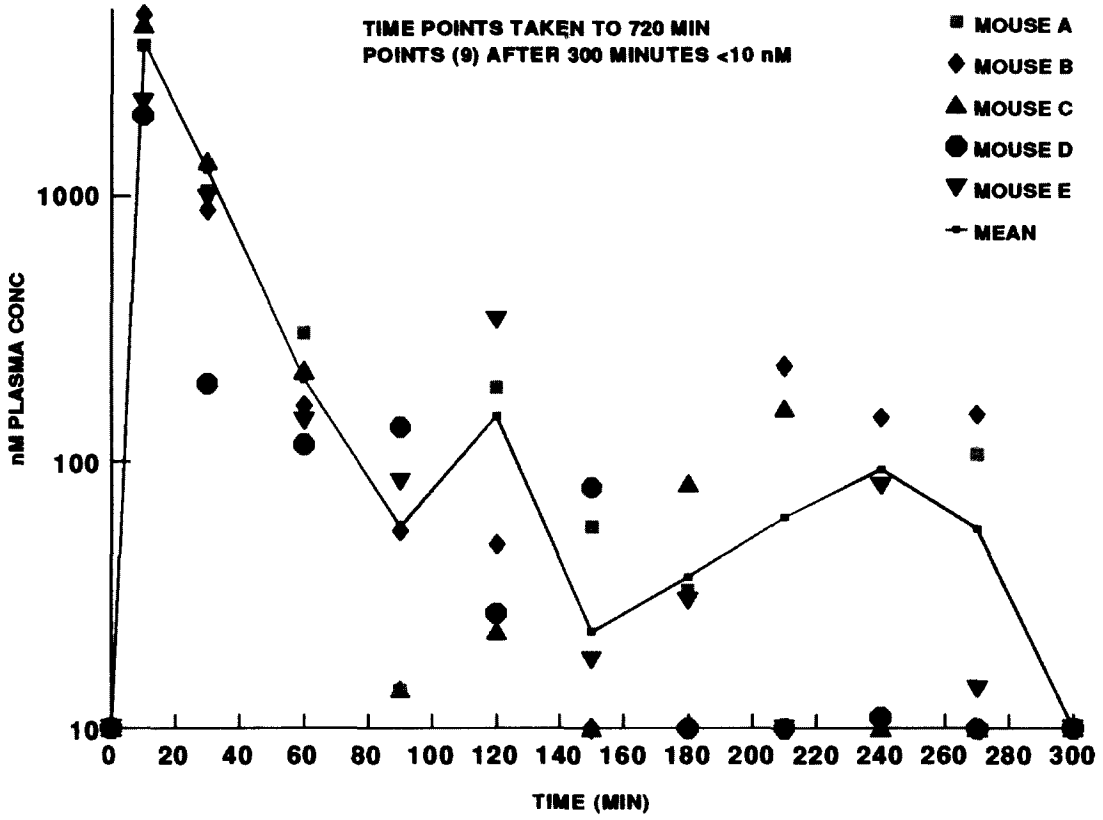

Fig. 3. Plasma levels of SKF 108922, formulated in HPB, after SC administration of a dose of 5 mg/kg in a volume of 0.1 ml. Drug levels were measured by inhibition of purified HIV-1 protease.

Table 1
Effect of protease inhibitors on erythroleukemia in RMuLV model

Treatment	Dose (mg/kg)	Erythroleukemia in spleen	Erythroleukemia in liver	Erythroleukemia in bone marrow
Uninfected		0	0	0
RMuLV + Saline		3	2	3
RMuLV + AZT	0.3 mg/ml (PO in DW)	0	0	0
RMuLV + HPB Vehicle		4	5	3
RMuLV + SKF 109273	2.2	5	5	4
	3.3	5	4	2
	6.7	4	5	2
RMuLV + SKF 108922	2.1	5	3	3
	4.4	1	1	0
	11	3	3	3

PO = oral DW = drinking water
Tissues collected from the mice in the experiment describe din Fig. 1 were preserved in buffered formalin. The preserved tissues were embedded in paraffin, sectioned, stained with hematoxylin-eosin, and evaluated microscopically (in blinded fashion) for evidence of leukemia. The extent of erythroleukemia was scored on the following scale: 0 = no erthroleukemia 1 = minimal 2 = mild 3 = moderate 4 = marked 5 = severe.

3.4. Circulating levels of SKF 108922 in plasma

Measurements of the circulating levels of the protease inhibitor in plasma by enzyme assay (Fig. 3) were consistent with these results. Within 10 min after SC administration of SKF 108922 solubilized in HPB at a dose of 5 mg/kg, plasma concentrations of the protease inhibitor exceeded 1000 nM in all mice. Within an hour, average plasma concentrations had dropped to ~ 100 nM, at which level they remained for 4–4.5 h. At 5 h after administration, levels of SKF 108922 were < 10 nM. In contrast, SKF 108922 was not detected in plasma after SC administration of the same dose formulated with cholesterol sulfate, instead of HPB (data not shown).

4. Discussion

The studies described in the present report confirm and extend our previous report (Black et al., 1993) of the antiretroviral activity of rationally designed synthetic protease inhibitors against RMuLV in vitro. We have previously presented a preliminary report of these findings at the VII International Conference on AIDS in Florence, Italy, in 1991 (Ussery et al., 1991), at which conference were reported similar studies of another protease inhibitor with a related virus, the Friend MuLV model (Lai et al., 1991). Our results are consistent with those reported by Lai et al. (1993), which have since been published.

SKF 108922, which inhibited HIV, SIV, and RMuLV in vitro (Black et al., 1993), also dramatically reduced infectious virus titer in the serum of RMuLV-infected mice in a dose-dependent manner. In contrast, SKF 109273, which lacked antiviral activity against the three retroviruses in vitro (Black et al., 1993), also failed to protect mice from RMuLV-induced disease in vivo. Thus, the antiretroviral effects observed in vivo in RMuLV-infected mice were entirely consistent with the relative activities of these two protease inhibitors in vitro.

Our data suggest that splenomegaly was only moderately reduced in animals treated with the active protease inhibitor as compared with AZT treatment. However, SKF 108922 effectively inhibited production of infectious virions in the serum of animals. Since splenomegaly was not inhibited dramatically, perhaps SKF 108922 did not inhibit production of non-infectious particles of RMuLV. Production of non-infectious particles (i.e., protease inhibitor treated) in these animals may be sufficient to allow replication of the defective virus (SFFV) particles (or merely the production of the SFFV oncogene, gp54) resulting in induction of erythropoiesis and subsequent splenomegaly. Once these processes have been initiated, antiviral therapy, no matter how effective, would not reverse them. Inhibition of viremia is thus a more meaningful measure of antiviral effects. Therefore, the discrepancy between inhibition of splenomegaly and viremia by the protease inhibitor is more apparent than substantive.

Indeed, Crawford and Goff (1985) demonstrated that Moloney MuLV containing a deletion in the 5′ region of *pol* (i.e., protease deletion) contained unprocessed $Pr65_{gag}$ and $Pr200_{gag\text{-}pol}$ but contained unexpectedly high levels of RT activity (dot blots suggested perhaps 33–50% of wild-type RT activity). The mutant virus was poorly infectious. A conclusion of this paper was that "cleavage of the *gag* gene product is not required for budding and release of virions and that complete processing of the *pol* gene product to the mature form of reverse transcriptase is not required for its functional activation." It is likely, therefore, that Rauscher MuLV will also behave similarly when protease is inhibited, yielding immature virions which have levels of RT activity similar to untreated controls. Indeed in our previous report (Black et al., 1993), we noted by electron microscopy the production of many virions with the altered morphologies characteristic of immature, non-infectious virus particles after treatment of RMuLV-infected cells with protease inhibitors. The nature of the RT activity in such immature particles is being investigated.

Besides the relatively modest inhibition of splenomegaly observed in these experiments, a more serious problem was the exacerbation of disease produced by the HPB vehicle used to solubilize the protease inhibitor. The excipient consistently increased splenomegaly 2–3-fold and increased viremia and serum RT levels by greater than 10-fold. It should be noted that HPB had no detectable effects on uninfected animals (data not

shown). Also, the enhancing effect of HPB was completely reversed by AZT, and animals treated with AZT in the presence of HPB (with or without protease inhibitors) were not significantly different (in terms of splenomegaly, viremia, or serum RT) from animals treated with AZT alone (data not shown). Anand et al. (1990) reported that sulfated α- and β-cyclodextrins stimulated the proliferation of human peripheral blood mononuclear cells and that β-cyclodextrin sulfate at low concentrations enhanced HIV replication in vitro. Our results suggest that hydoxypropyl derivatives of cyclodextrin, at the concentrations tested, have similar lymphoproliferative effects in vivo. Thus, derivatized cyclodextrins may not be suitable for delivering protease inhibitors to patients.

In fact, we have reported in this communication some preliminary studies on alternative vehicles and routes of drug administration. Administration of SKF 108922 by the SC route had a similar antiviral effect as the same dose given by the IP route. However, changing the formulation from HPB to cholesterol sulfate eliminated the antiviral effect of SKF 108922 (administered SC). Thus, changing the vehicle for administering the protease inhibitor had a considerable effect on antiviral activity, while changing the route of administration did not in these limited studies. Clearly, further work on formulation of protease inhibitors and on the design of more water-soluble inhibitors is necessary.

Measurements of the circulating levels of SKF 108922 in plasma explain the difference between the antiviral activities seen with the two vehicles. Within 10 min after SC administration of SKF 108922 solubilized in HPB, plasma concentrations of the protease inhibitor exceeded 1000 nM in all mice (Fig. 3). Within an hour, average plasma concentrations fell to $\sim$ 100 nM, at which level they remained for 4–4.5 h. At 5 h after administration, levels of SKF 108922 dropped below 10 nM. In contrast, SKF 108922 was not detected in plasma after SC administration of the same dose delivered as a colloidal dispersion in cholesterol sulfate, instead of HPB. It is possible that uptake of SKF 108922 in the colloidal dispersion may have occurred predominantly by the draining lymph nodes, but levels of SKF 108922 in lymph were not measured in these studies. The peak plasma level of SKF 108922 after SC administration of SKF 108922 (5 mg/kg) solubilized in HPB exceeded the IC_{50} (1000 nM) and IC_{90} values (1800 nm) observed in vitro for RMuLV (Black et al., 1993). Average plasma levels of SKF 108922 remained at this level ($\geq$ 1000 nM) for only 30 min, but remained at about 100 nM for 4–4.5 h after administration. Administration of protease inhibitor more frequently than twice a day may be necessary to maintain therapeutic levels in circulation and should improve antiviral activity.

In summary, the results presented here demonstrate that the protease inhibitor SKF 108922 has antiviral activity in vivo in the RMuLV retrovirus model. These studies show that sufficient circulating levels of SKF 108922 can be attained in mice to affect the course of RMuLV disease. Therefore, RMuLV should provide a good model for the preclinical evaluation and development of this class of therapeutic agents for HIV (in the case of inhibitors with similar activities against RMuLV and HIV-1 proteases). Meaningful and convenient animal models could permit optimization of the conditions for administering protease inhibitors. Although RMuLV does not represent a realistic model of HIV pathogenesis, it can provide valuable guidance in areas of formulation, route of delivery, and bioavailability. Such information should prove especially useful in guiding the clinical application of protease inhibitors.

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