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Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *Salvia miltiorrhiza*

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Abstract

Water soluble extracts of the herbal plant, *Salvia miltiorrhiza* (Danshen) exhibited potent effect against HIV-1 integrase activity in vitro and viral replication in vivo. We have developed an extensive purification scheme to isolate effective, non-toxic inhibitors against human immunodeficiency virus type 1 (HIV-1) using the 3′-processing activity of integrase as a purification guide and assay. Two water soluble compounds, $M_5$22 and $M_5$32, have been discovered by isolating them from *S. miltiorrhiza* roots in purities of $>99.5\%$ as shown by NMR spectral analysis with yields of 0.018 and 0.038%, respectively. Structural determination revealed that $M_5$22 is lithospermic acid and $M_5$32 is lithospermic acid B. These two structurally related compounds are potent anti-HIV inhibitors and showed no cytotoxicity to H9 cells at high concentrations ($CC_{100} > 297$ μM for $M_5$22 and > 223 μM for $M_5$32). The IC_{50} for inhibition of 3′-processing by HIV-1 integrase was found to be 0.83 μM for $M_5$22 and 0.48 μM for $M_5$32. In addition, $M_5$22 and $M_5$32 inhibited HIV-1 integrase catalytic activities of 3′-joining to the target DNA with IC_{50} of 0.48 μM for $M_5$22 and 0.37 μM for $M_5$32. Furthermore, kinetic and mechanistic studies suggested that drug binding to HIV-1 integrase and inhibition of enzymatic activity occur at a fast rate. Both $M_5$22 and $M_5$32 do not prevent HIV entry in H9 cells. They also show no inhibition of reverse transcriptase activity in infected cells. The levels of intracellular strong stop and full-length viral DNA remained unchanged following drug treatment. However, both inhibitors strongly suppressed the acute HIV-1 infection of H9 cells with IC_{50} values of 2 and 6.9 μM for $M_5$22 and $M_5$32, respectively. Thus these two selective integrase inhibitors hold promise as a novel class of therapeutic drugs for AIDS based on their high potencies and absence of cytotoxicity.

Keywords: HIV-1; Inhibition; Purification; HPLC; HIV-1 integrase; Inhibitors

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1. Introduction

The human immunodeficiency virus type 1 (HIV-1) integrase (Goff, 1992) is an enzyme that mediates the integration of HIV-1 DNA into the host chromosome and is essential for replication of the virus and is required for stable and productive infection (Engelman et al., 1995; LaFemina et al., 1992; Roth et al., 1990; Sakai et al., 1993; Shin et al., 1994). It has no counterpart in the host cell and is therefore, a suitable target for drug therapeutic intervention and it has become a focus of anti-AIDS drug design. Following reverse transcription of the viral RNA genome into a double-stranded DNA (dsDNA), integrase (IN) catalyzes integration of the latter into the host chromosome through coordinated reactions of processing and joining (Brown et al., 1989). Initially the enzyme recognizes the LTR termini of the viral dsDNA, and removes the last two nucleotides (GT) leaving two recessed 3′-OH ends. Then integrase catalyzes joining of the processed 3′-ends of the virus to the 5′-ends of strand breaks in the host DNA. Removal of mispaired nucleotides and gap repair, which are carried out by cellular enzymes, lead to provirus formation (Bushman et al., 1990).

A well known medicinal plant, *Salvia miltiorrhiza* (Danshen) has been used extensively for the treatment of many diseases and is believed to have antioxidative capabilities (Liu et al., 1999). Two constituents of this medicinal plant, cryptotanshinone and dihydrotanshinone I, have been shown to have antibacterial activity against a broad range of Gram positive bacteria (Lee et al., 1999) and to inhibit mast cell degranulation significantly (Ryu et al., 1999). It has also been observed that five compounds isolated from *S. miltiorrhiza* can inhibit the activity of unsaturated fatty acids and anacardic acids toward the soluble factor–factor Vlla complex (Wang et al., 1998). This plant has received much interest due to its ability to accumulate large amounts of active natural products such as tanshinones (Chen et al., 1997) and phenolic compounds (Hase et al., 1997; Kamata et al., 1993, 1994; Morimoto et al., 1994; Tanaka et al., 1989). The antioxidant activity of *S. miltiorrhiza* phenolics, which is generally ascribed to their hydroxyl groups (Chen and Ho, 1997), may have potential benefits for human health (Pearson et al., 1997). Seven phenolic compounds isolated from an aqueous extract of *S. miltiorrhiza* demonstrated a strong protective action against peroxidative damage to liver microsomes, hepatocytes, and erythrocytes (Liu et al., 1992). The dry roots of *S. miltiorrhiza* have been used in Chinese folk medicine for the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction (Chinese Medicinal Dictionary, 1977). In addition to these therapeutic activities, it has been found that a crude extract from one species of Danshen (*Salvia yunnanensis*) contains antiviral activity of HIV-1 reverse transcriptase and integrase in vitro. This crude extract inhibited p24 expression in human PBMC cultures infected with AZT sensitive and resistant HIV-1 and showed synergistic activity with AZT against AZT resistant HIV-1 strains. The crude extract when administered orally protected mice from murine leukemia virus (MuLV) infection and monkeys from SIV infection (Chen et al., 1997, abstract of 11th International Conference on Antiviral Research).

In this report, we described the purification steps for isolation of active anti-HIV compounds from crude extracts of *S. miltiorrhiza* roots. Two potent, non-toxic HIV-1 integrase inhibitors, $M_5$22 and $M_5$32, were isolated by using high performance liquid chromatography (HPLC). Both are pure compounds, which showed strong anti-HIV activity in infected H9 cells. Chemical synthesis of $M_5$22 and $M_5$32 is currently in progress and studies of the inhibition of HIV-1 replication by these inhibitors in animals are planned.

2. Materials and methods

2.1. Isolation of Bu.M_5 fraction from *S. miltiorrhiza* roots

Root powder (1.5 kg) of *S. miltiorrhiza* (supplied by Jiang Su Danhua Group Co., China) was extracted three times with 4.5 l of 70% methanol for 24 h each time with vigorous shaking. The three extracts (70 S fraction) were combined,

evaporated to dryness and dissolved in 2.0 l of 25% NH_4OH. The extract solution was then precipitated with four volumes of 1-propanol and the precipitate was washed three times with 5.0 l of P.8 buffer (Propanol–H_2O–NH_4OH, 80:15:5). Both the propanol soluble (P.8s) and precipitate (P.8p) fractions were tested for anti-HIV integrase activity. For further purification of the P.8p fraction, 50 g were dissolved in 200 ml of NAA buffer (7% acetonitrile, 0.1 M of ammonium acetate; 0.5% acetic acid) and the cleared NAA fraction was next mixed with 200 ml of 1-butanol and shaken vigorously. The mixture was partitioned into an NAA layer and a butanol layer, which were collected separately. The butanol fraction was dried, redissolved in 5% methanol, cleared by centrifugation and then dried again (Bu.M_5). The anti-HIV integrase activity of Bu.M_5 was also tested.

2.2. Testing the toxicity of Bu.M_5 fractions in mice

The toxicity of the Bu.M_5 fraction was tested in C57bl/6 mice by tail vein injections of the Bu.M_5 fraction (2 mg/ml in 0.9% NaCl) over a period of 7 days at a daily dose of 10, 20 and 30 mg/kg range, using two mice per each dosage group.

2.3. HPLC method for isolation of $M_5$22 and $M_5$32 from the Bu.M_5 fraction

The last step of purification of the HIV-1 inhibitors was carried out using high performance liquid chromatography (HPLC) with a limited amount (100 mg) of butanol fraction (Bu.M_5) for each run. HPLC was performed on Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The extract solution was separated and analyzed by using a 250×10 mm preparative C18 (8 μm) column with the mobile phase consisting of 5% methanol. The flow rate was 1.0 ml/min and the elution was monitored at a wavelength of 254 nm to facilitate the detection of the different compounds. Two major peaks of Bu.M_5 fraction were well separated from each other and from the rest of the mixture. These two major peaks were individually collected and reapplied to the column to achieve $>99.5\%$ purity of the compounds. Approximately, 500 of HPLC runs were made to isolate sufficient amount of $M_5$22 and $M_5$32 for antiviral integrase studies.

2.4. HIV-1 integrase assays

2.4.1. 3′-End processing

DNA substrate for 3′-end processing assay consisted of a DNA sequence derived from the U5 end of HIV-1 LTR. It was prepared by annealing oligonucleotide U5V1 (5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT) with its complementary strand U5V2 (3′-CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA). The U5V1 strand was labeled at the 5′-end with [γ-^{32}P] ATP using T4 polynucleotide kinase as described previously (Kamata et al., 1994). The integrase reaction was a modification of the method described by Chow (1997). The reaction mixture included 40 mM HEPES, pH 7.5, 20 mM $MnCl_2$, 60 mM NaCl, 20 mM DTT, 0.1% Nonidet-P40 and HIV-1 integrase at a final concentration of 300 nM (NIH AIDS Research and Reference Reagent Program). To assay for 3′-end processing, labeled substrate (65 nM per reaction) was incubated with the reaction mixture for 60 min at 37 °C. An equal volume of stop solution (95% formamide, 30 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to each reaction and the samples were heated to 95 °C for 5 min to denature the DNA. The samples were then fractionated by electrophorsis on a 15% denaturing polyacrylamide gel. The 3′-end processing activity was monitored by the appearance of a radioactive oligonucleotides product (33nt), shortened by two nucleotides from the original substrate (35nt). The products were visualized by autoradiography and quantitation was carried out by phosphorimaging. The percent inhibition was calculated using this equation: $100 \times [1 - (D - C)/(N - C)]$, where C, N, and D are the fractions of DNA substrate converted to product for DNA alone (C), DNA plus integrase (N), and integrase plus drug (D). Then the IC_{50} was determined by plotting the log of drug concentration versus percent inhibition

and determine the concentration which produces 50% inhibition.

2.4.2. 3′-End joining

To assay only the 3′-end joining activity independent of 3′-end processing, a substrate that resembles the viral U5 end of HIV-1 LTR after 3′-end processing was used. The following oligonucleotides were purchased from MWG-Biotech Inc. The oligonucleotides were purified by high-performance liquid chromatography (HPLC). The preprocessed double strand DNA (33nt/35nt) substrate was prepared by annealing the labeled strand U5V1 (5′-GACCCTTTTAG-TCAGTGTGGAAAATCTCTAGCA) with its complementary strand U5V2 (3′-CTGGGAAA-ATCAGTCACACCTTTTAGAGATCGTCA) as described above for the 3′-end processing assay. The joining activity is assayed by the appearance of labeled products that are longer than the input DNA on a denaturing gel. The length of the products are heterogenous because the site of joining is largely random. The products were visualized by autoradiography and quantitation was carried out by phosphorimaging.

2.5. Kinetic and mechanistic inhibition by $M_5$22

To test the binding rate of $M_5$22 with HIV-1 integrase, we examined the extent of 3′-processing catalyzed by integrase when the inhibitor $M_5$22 was or was not pre-incubated with HIV-1 integrase. The concentration of inhibitor that used in these studies was 0.45 μg/ml, which is the IC_{50} of $M_5$22. The method was performed as described previously (Mazumder et al., 1997). HIV-1 integrase (300 nM) was pre-incubated with $M_5$22 for 30 min at 37 °C in reaction buffer (see Section 2.4). Then, the labeled viral DNA substrate (65 nM) was added and the reaction continued for additional 26 min at 37 °C. An equal volume of stop solution was added as described above and the samples were then fractionated by electrophorsis on a 15% denaturing polyacrylamide gel. The products were visualized by autoradiography and quantitation was carried out by phosphorimaging.

2.6. Cytotoxicity assay

The cytotoxicity of $M_5$22 and $M_5$32 drugs against H9 cells was analyzed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Chemical Co.) (Uckun et al., 1998). Briefly, exponentially growing H9 cells were seeded onto 96-well plates at a density of 3×10^4 cells/well and incubated for 24 h at 37 °C prior to drug exposure. On the day of treatment, a series of $M_5$22 and $M_5$32 concentrations (1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/ml) were used to test their cytotoxicity in H9 cells. Quadruplicate wells were used for each treatment. The cells were incubated with $M_5$22 and $M_5$32 for 4 days at 37 °C in a humidified 5% CO_2 atmosphere. H9 cells were subcultured with fresh culture medium containing appropriate concentrations of $M_5$22 and $M_5$32 and further incubation till day 8 of drug treatment. To each well, 50 μl of MTT was added (1 mg/ml final concentration) and the plates were incubated at 37 °C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized with DMSO. The absorbance of each well was measured in a microtiter reader at optical density 540 nm. The reaction is specific, no significant amounts of formazan can be detected with dead cells.

2.7. Cells, virus strains and antiviral assay

The following cells and virus strain were obtained from the AIDS Research and Reference Regent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: H9 cells, virus strains of HIV-1_{IIIB}A17, a variant that is highly resistant to RT non-nucleoside inhibitors. H9 cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. H9 cells were incubated with HIV-1 virus at 37 °C in CO_2 for 2 h. H9 cells were suspended in culture medium at 1×10^5 cells/ml and infected with HIV at a multiplicity of infection of 0.1. After infection, the cells were washed twice with PBS followed by culture medium. The cell suspension (100 μl) was added

to each well of 96-well plate and then various concentrations of $M_5$22 and $M_5$32 were added to the cells infected with HIV-1_{IIIB}A17. After a 4-day incubation at 37 °C, H9 cells were subcultured with fresh culture medium containing appropriate concentrations of $M_5$22 and $M_5$32 and further incubation till day 8 after infection. Viral replication was determined by the amount of HIV-1 p24 antigen in culture supernatant on days 4 and 8 after virus infection of H9 cells using the HIV/p24 monoclonal antibody assay (Veronese et al., 1985).

2.8. Virus-binding assay

H9 cells (4×10^5) were infected with HIV-1_{MN} virus in the presence or absence of various concentrations of $M_5$22 and $M_5$32 drugs and incubated for 1 h at 37 °C to permit virus fusion and entry. Then, the cells were centrifuged and washed three times with PBS to remove the unbound virus as described previously (Este et al., 1998). The viral entry was determined by p24 antigen-capture assay (Veronese et al., 1985).

2.9. PCR analysis for infected cells

H9 cells were used to determine the reverse transcriptase activity of HIV-1 following infection by measuring the intracellular strong stop viral DNA by using the methods described previously (Simm et al., 1996; Zack et al., 1990). H9 cells (1×10^6) were infected with HIV-1_{MN} in presence of various concentrations, 0, 1.25, 10, 20 μg/ml of $M_5$22 and $M_5$32 drugs and incubated for 1 h at 37 °C in CO_2 to permit virus fusion and entry. Then, the cells were centrifuged and washed three times with PBS to remove the unbound virus. H9 cells were lysed in PCR lysis buffer (Inis et al., 1990) containig 10 mM Tris–HCl, pH 8.3, 1 mM EDTA, 0.5% Triton X-100, 0.001% sodium dodecyl sulfate, and 300 μg of proteinase K/ml. The samples were incubated at 55 °C for 1 h and then boiled for 15 min. Samples were subjected to phenol–chloroform extraction and ethanol precipitation. PCR was performed using DNA from 1×10^6 cells and specific primers to detect two regions of viral DNA synthesized at different stages of reverse transcription: the R/U5 region of the long terminal repeat (strong stop DNA), which is amplified from both incomplete and full-length viral transcripts, and the long terminal repeat/gag region, which is amplified only from full-length transcripts (Zack et al., 1990). The strong stop primers were (5′-GGCTAACTAGGGAACCCACTG-3′, nucleotides 495–515) and (5′-CTGCTAGAGATTTTCCACACTGCC-3′) nucleotides 611–634) yield a PCR product of 140 base pairs, and the full-length DNA primers were (5′-GAGTCCTGCGTCGAGAGAGCTCTG-3′, nucleotides 672–695) and (5′-GGCTAACTAGGGAACCCACTG-3′, nucleotides 495–515) which should give a 200 base pairs of PCR product. PCR was performed by using 10 μM of each primer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA Polymerase (Invitrogen) for 40 cycles of denaturation time (94 °C for 30 s), annealing (60 °C for 30 s) and extension (72 °C for 30 s). Amplified products resulting from the PCR were analyzed by electrophoresis on 6% non-denaturing polyacrlyamide gel and visualized by ethidium bromide staining.

3. Results

3.1. Isolation of anti-HIV inhibitors $M_5$22 and $M_5$32 from S. miltiorrhiza roots

Three major purification steps were employed for the isolation of pure compounds, $M_5$22 and $M_5$32. Step one was a preparative one, in which 70% methanol was used to extract material from a large quantity (1.5 kg) of the dried roots and a P.8 buffer ((Propanol–H_2O–NH_4OH) with the ratio of (80:15:5)) was used to selectively precipitate the anti-HIV compounds from the 70% methanol extract (70 S). The P.8 precipitates (P.8p), which represented approximately 34% of the root material, showed potent anti-HIV integrase activity in the 3′-cleavage activity assay with an IC_{50} of 0.1 μg/ml, while the P.8 soluble fraction (P.8s), representing 66% of the starting

Table 1
A summary of the purification of HIV-1 inhibitors from *S. miltiorrhiza* roots

	Fraction	Amount (g)	Yield (%)
	S. miltiorrhiza roots	1500	
Step I	P.8p	516	34.4
Step II	Bu.M_5	2.2	0.15
Step III	HPLC pure compounds		
	$M_5$22	0.277	0.018
	$M_5$32	0.572	0.038

material was relatively less active ($IC_{50} = 1$ μg/ml). Although, the P.8p fraction contained highly active HIV integrase inhibitors, it also contained materials toxic to animals as indicated by our toxicity tests in mice (data not shown). To separate these toxic materials from the anti-viral components, a second semi-preparative step was employed. This second step consisted of a two-phase separation system with NAA buffer (7% acetonitrile, 0.1 M of ammonium acetate, 0.5% acetic acid) as the aqueous layer and 1-butanol as the organic layer. Anti-HIV integrase activity (IC_{50} is 0.6 μg/ml) was recovered from the combined butanol extracts (Bu.M_5). Although, the yield of the BuM_5 is poor, this fraction showed no toxicity in C57bl/6 mice, with no weight loss observed after tail vein injections of 10–30 mg/kg each day for 7 days (data not shown). High performance liquid chromatography (HPLC) was used to further purify the Bu.M_5 fraction and to isolate pure anti-HIV compounds from *S. miltiorrhiza* roots. Two major peaks with retention times of 22.4 and 31.4 min ($M_5$22 and $M_5$32) were well separated from a large, exceedingly complex mixture of unresolved compounds. $M_5$22 and $M_5$32 were pooled separately and then reapplied to the column. Homogenous $M_5$22 and $M_5$32 preparations were recovered after second HPLC purifica-

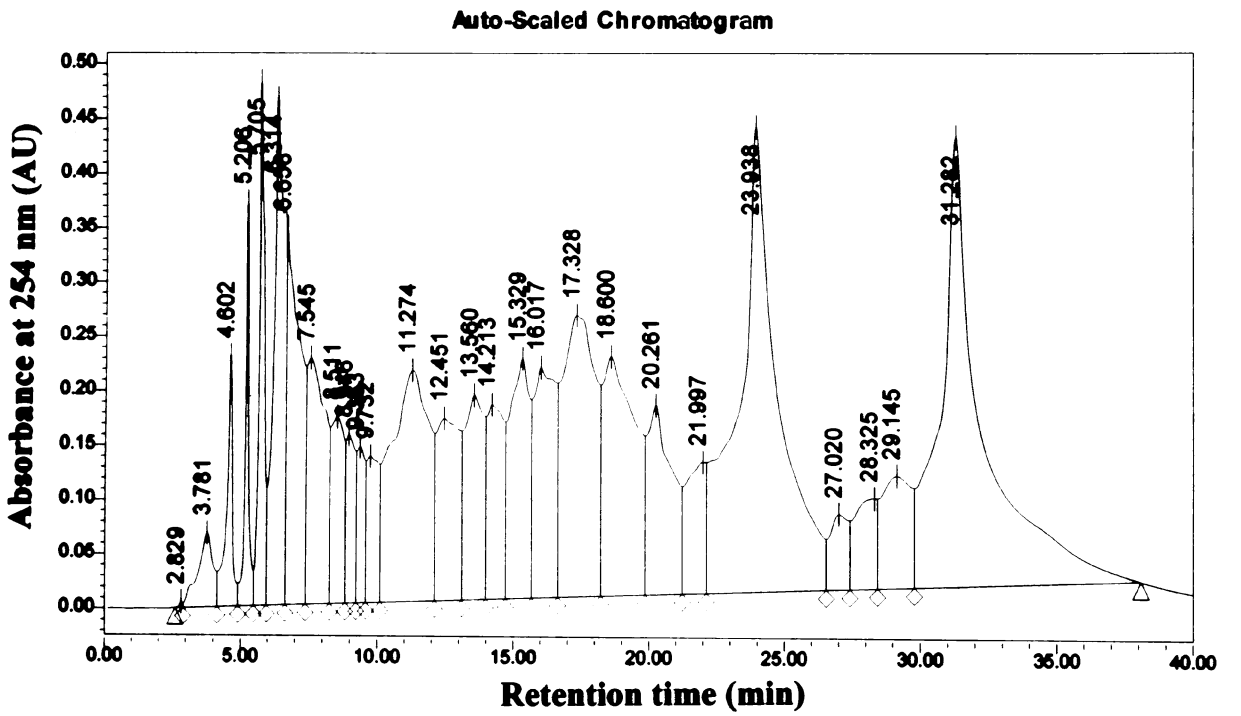


Fig. 1. HPLC chromatogram of the Bu.M_5 fraction. Many peaks were resolved at different retention times. The activity of each one was tested against HIV-1 integrase activity. The peaks at retention times 23.038 ($M_5$22) and 31.282 ($M_5$32) were identified as the major active fractions.

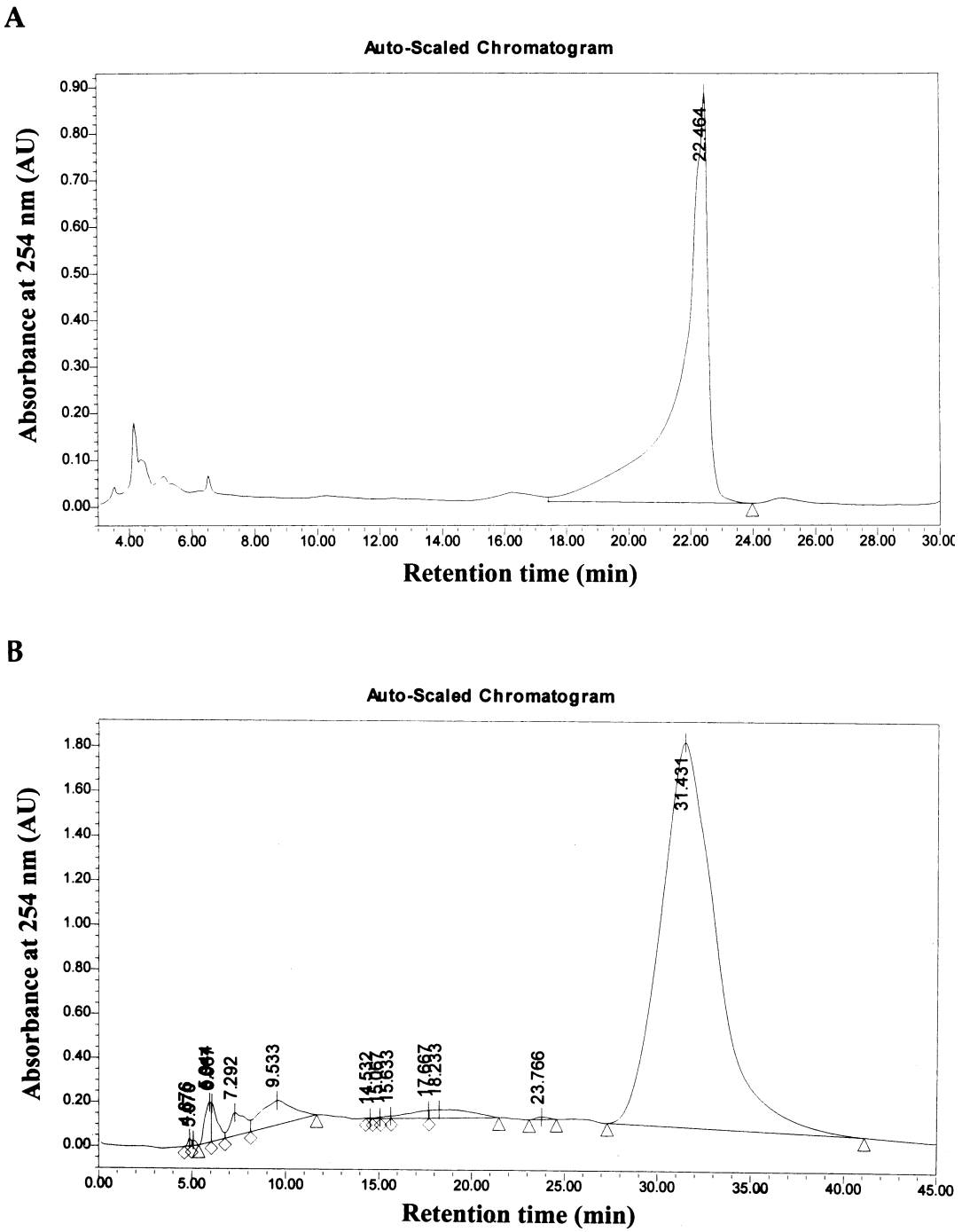
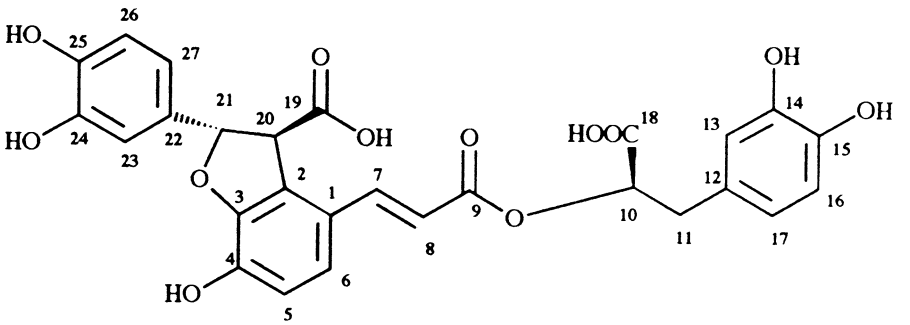


Fig. 2. HPLC chromatogram of separately pooled $M_5$22 and $M_5$32 peaks from Fig. 1 and reapplied to the column. (A) This figure shows a single and major peak ($M_5$22) was eluted at 22.4 retention time. (B) This figure shows one major peak ($M_5$32) that eluted at about 31.4 retention time. Both $M_5$22 and $M_5$32 were found to be pure compounds by NMR analysis.

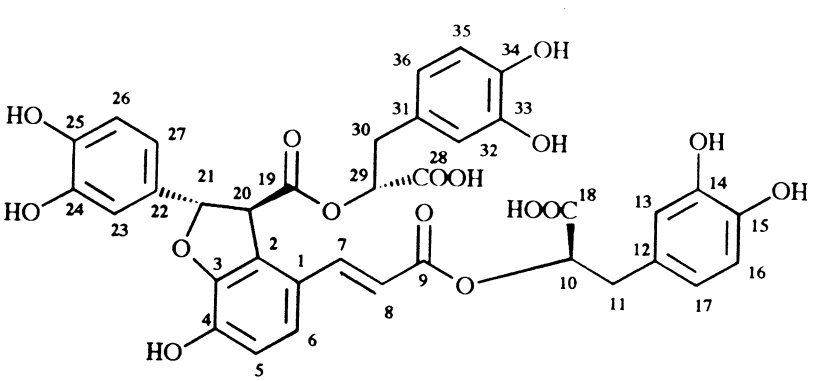
A



$M_5$22 (Lithospermic acid)

(4-{2-[1-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid)

B



$M_5$32 (Lithospermic acid B)

(4-{2-[1-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester)

Fig. 3. Chemical structures of compounds $M_5$22 and $M_5$32 inhibitors. (A) $M_5$22 (lithospermic acid). (B) $M_5$32 (lithospermic acid B).

tion. The yield for $M_5$22 and $M_5$32 from the Bu.M_5 fraction was approximately 13 and 26%, respectively. An over all yield from the initial *S. miltiorrhiza* roots of 0.018 and 0.038% for $M_5$22 and $M_5$32, respec-tively was obtained (Table 1). The HPLC profiles for compounds $M_5$22 and $M_5$32 are shown in Figs. 1 and 2.

3.2. Identification of the structures of $M_5$22 and $M_5$32 and analyses of their anti-HIV efficacies and their cellular toxicities

The purity of the two compounds, $M_5$22 and $M_5$32, was assessed by NMR analysis. Each individual 500 MHz dimethylsulfoxide-d_6 ^{1}H NMR spectrum revealed no contaminating peaks from the other compound. In addition, no peaks unaccounted for from the structural determination of each compound were found (Bates, 2001, NMR profile record, 2001). From these data, we concluded that our isolated samples, $M_5$22 and $M_5$32 are $>99.5\%$ pure. The structures of $M_5$22 and $M_5$32 were identified by NMR and MS analysis using known structural standards for comparison (Tanaka et al., 1989). $M_5$22 was identified as lithospermic acid (4-{2-[1-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid) (Fig. 3A) and $M_5$32 as lithospermic acid B (4-{2-[1-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3 dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester) (Fig. 3B). Their anti-HIV efficacies were analyzed by their effect on the 3′-processing activity and their inhibition of HIV in cultured H9 cells. The inhibitory effect of $M_5$22 and $M_5$32 against HIV replication was examined using HIV-1_{IIIB}A17, a variant that is highly resistant to inhibition by RT non-nucleoside inhibitors with $M_5$22 and $M_5$32, as testing virus in the presence of a variety of drug concentrations in infected H9 cells. By using the HIV/p24 monoclonal antibody assay, the IC_{50} (50% inhibitory concentration) for $M_5$22 and $M_5$32 against HIV replication were found to be 1.1 μg/ml, 2 μM for $M_5$22 and 5 μg/ml, 6.9 μM for $M_5$32, respectively (Fig. 4A). A series of drug concentrations ranging from 1.25 to 160 μg/ml was used for testing the cytotoxicity of $M_5$22 and $M_5$32 in H9 cells. Using MTT assay to determine the viability of H9 cell in culture, we found that the cells remained viable during the entire culture period of 8 days even at the highest concentrations (CC_{100}) of $M_5$22 and $M_5$32 tested (160 μg/ml, 297 μM for $M_5$22 and 160 μg'ml, 223 μM for $M_5$32) (Fig. 4B). The inhibitory data that

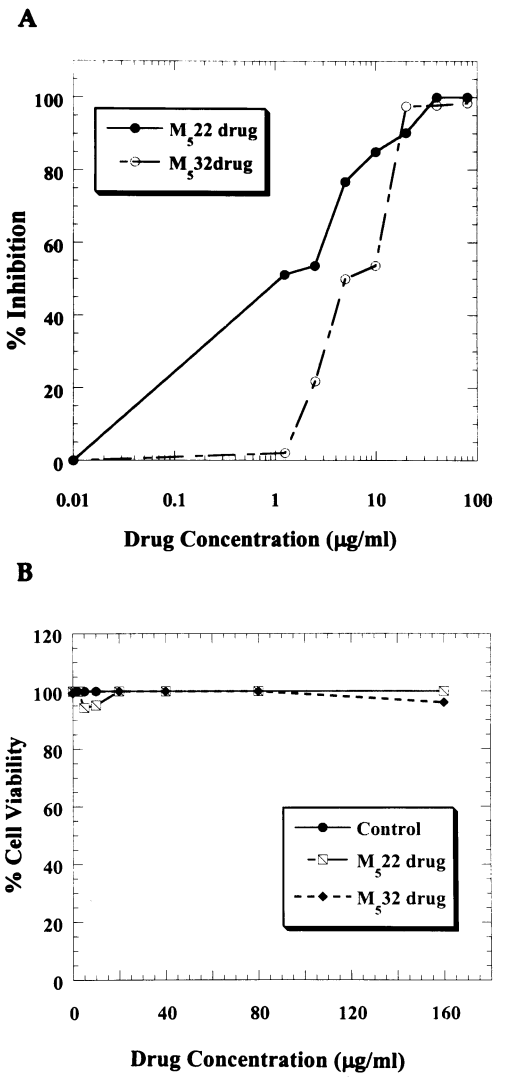


Fig. 4. Effect of $M_5$22 and $M_5$32 on HIV-1 replication and cell viability. (A) Dose-dependent inhibition of HIV-1 replication in H9 cells infected with HIV-1_{IIIB}A17 virus, a variant that is highly resistant to inhibition by RT non-nucleoside inhibitors, in the presence of various drug concentrations of $M_5$22 and $M_5$32. Viral replication was measured using the HIV/p24 monoclonal antibody assay and the IC_{50} values for inhibition of HIV-1_{IIIB}A17 by $M_5$22 and $M_5$32 against HIV replication were calculated. (B) Effect of $M_5$22 and $M_5$32 on H9 cell viability with various drug concentrations, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μg/ml. MTT assay was used to determine the cytotoxicity of $M_5$22 and $M_5$32 in H9 cells in culture.

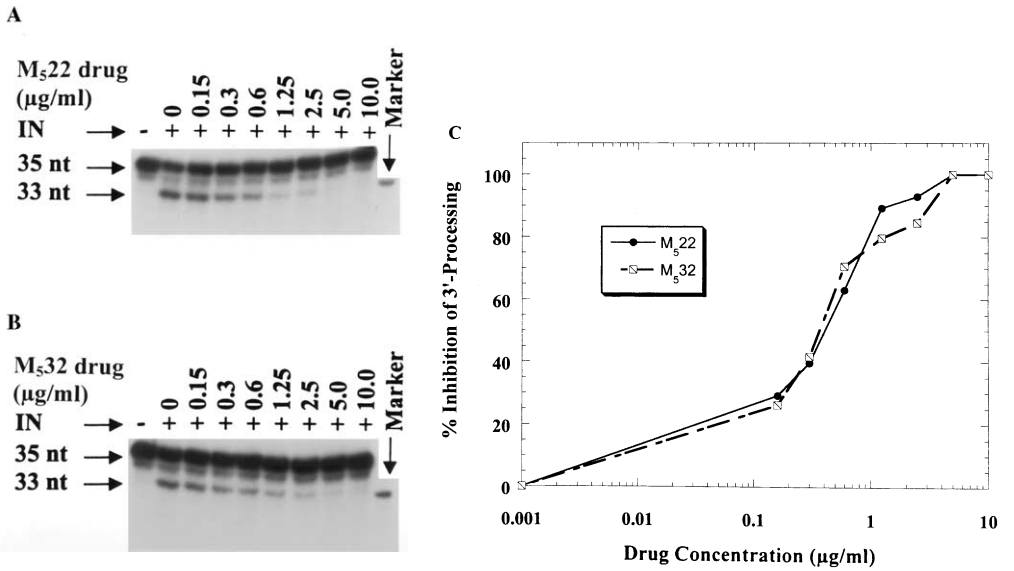


Fig. 5. Inhibition of 3′-end processing of HIV-1 integrase (IN) activity by $M_5$22 and $M_5$32. (A) Assay of inhibition by $M_5$22 drug. The 3′-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown. From left to right, lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3–9) integrase with 0.15, 0.3, 0.6, 1.25, 2.5, 5.0 and 10.0 μg/ml of $M_5$22 and lane 10, 33 nucleotide marker. (B) Assay of the inhibition by $M_5$32 drug. The 3′-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown. Lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3–9) integrase with 0.15, 0.3, 0.6, 1.25, 2.5, 5.0 and 10.0 μg/ml of $M_5$32 and lane 10, 33 nucleotide marker. (C) Quantitation of assays result by phosphorimager showing a dose–response curve for the inhibition of HIV-1 integrase activity by $M_5$22 and $M_5$32 drugs.

we have obtained, indicate that they are highly potent against HIV integrase, with IC_{50} of 0.45 μg/ml, 0.83 μM for $M_5$22 and 0.35 μg/ml, 0.45 μM for $M_5$32 (Fig. 5). In addition to examine their 3′-processing activities, (Fig. 5) and to prove these compounds are unique HIV integration inhibitors, we further tested the effect of $M_5$22 and $M_5$32, on the 3′-joining (strand transfer) which is the second function of the HIV-1 integrase that involves joining this recessed 3′-end to the 5′-end of an integrase-induced break in another identical oligonucleotide, as a target DNA. We found that both $M_5$22 and $M_5$32 inhibited of 3′-joining (or integration) of the recessed 3′-OH ends of the viral DNA to the 5′-P ends of the target DNA strongly with the IC_{50} of 0.26 μg/ml, 0.48 μM for $M_5$22 and 0.27 μg/ml, 0.37 μM for $M_5$32 (Fig. 6).

3.3. Kinetic and mechanistic inhibition by $M_5$22

We examined the extent of 3′-processing catalyzed by HIV-1 integrase when the inhibitor $M_5$22 was or was not preincubated with integrase. To test the effect of binding rate of $M_5$22 with HIV-1 integrase, we used in these studies, 0.45 μg/ml which is the IC_{50} of $M_5$22. In Fig. 7, the control of HIV-1 integrase exhibited a linear increase in the extent of 3′-processing over a time range of 0–26 min. When $M_5$22 inhibitor was pre-incubated with the enzyme without DNA substrate for 30 min, we found that the extent of 3′-processing of HIV-1 integrase was about 50% of the uninhibited control, exactly as found for $M_5$22 without pre-incubation with integrase prior to the start of the reaction. The slope of the line suggested that the inhibition of 3′-processing resulted from drug binding during the preincubation period and that there is a fast binding of $M_5$22 inhibitor to HIV-1 integrase.

3.4. $M_5$22 and $M_5$32 do not affect HIV-1 entry

To further prove that $M_5$22 and $M_5$32 only affect HIV integration processes, we examined whether $M_5$22 and $M_5$32 had any effect on viral entry to H9 cells. After 1 h incubation of cells with HIV-1_{MN} virus in presence of various concentrations, 0, 1.25,

2.5, 5, 10, 20, 40 and 80 μg/ml, the amount of virus P24 antigen was measured. We observed the same amount of HIV p24 detected in H9 cells both in the presence and absence of the drug added. Thus $M_5$22 and $M_5$32 are not viral entry inhibitors (Fig. 8).

3.5. $M_5$22 and $M_5$32 do not affect strong stop and full-length viral DNA synthesis

To show also that $M_5$22 and $M_5$32 are not inhibitory to reverse transcription activity, we analyzed viral DNA synthesis by PCR using primers to detect strong stop and full-length double-stranded viral DNA forms. Strong stop DNA represents R/U5 region of the viral long terminal repeat, which is synthesized first during reverse transcripition. Full-length viral DNA represents nearly completely synthesized viral DNA as amplified and detected by using LTR/gag primers. To test whether there is a possible inhibitory effect of $M_5$22 and $M_5$32 on synthesizing strong stop and full-length viral DNA, H9 cells were incubated with HIV-1_{MN} virus in presence of various concentrations, 0, 1.25, 10, 20 μg/ml for 1 h at 37 °C (Fig. 9). We found there is no any effect of these drugs on synthesizing strong stop or full-length double-stranded viral DNA.

4. Discussion

The therapeutic value of lithospermic acid B has previously been demonstrated in several studies. These benefical effects include hepatoprotec-

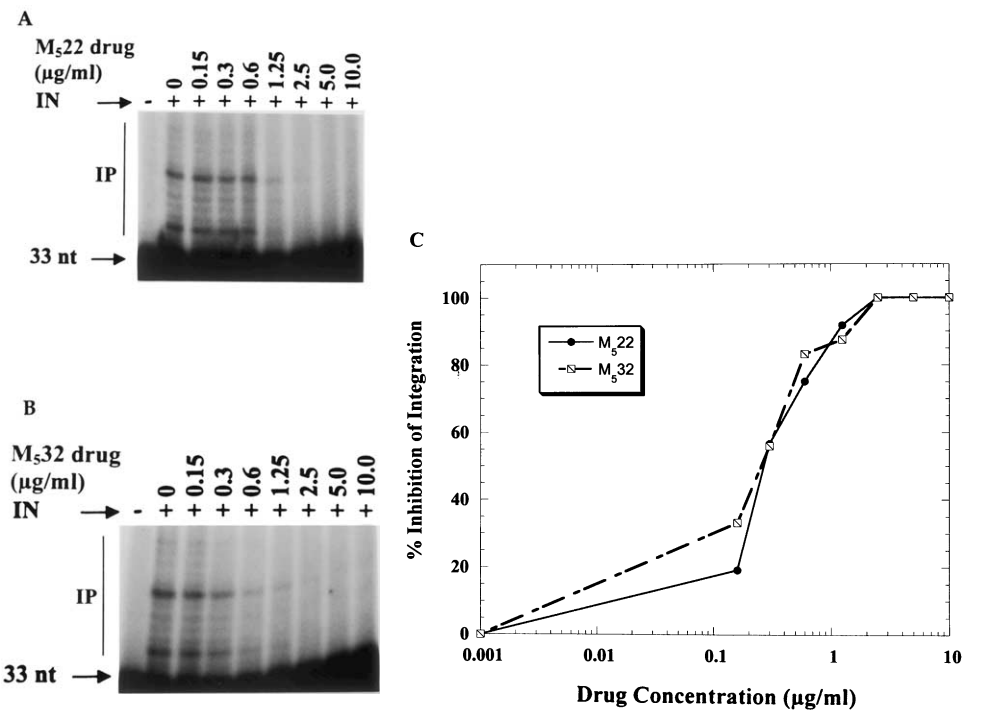


Fig. 6. Inhibition of 3′-end joining (strand transfer) of HIV-1 integrase (IN) activity by $M_5$22 and $M_5$32 drugs. The joining activity is assayed by appearance of products that are larger than the input DNA. The length of the products are heterogenous because the sites of joining is largely random. (A) Inhibition of catalytic function of HIV-1 integrase for 3′-joninig to the target DNA by $M_5$22. Where DNA substrate is 33nt and IP is integration products. Lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3–9) integrase with 0.15, 0.3, 0.6, 1.25, 2.5, 5.0 and 10.0 μg/ml of $M_5$22. (B) Inhibition of catalytic function of HIV-1 integrase for 3′-joninig to the target DNA by $M_5$32. Where DNA substrate is 33nt and IP is integration products. Lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3–9) integrase with 0.15, 0.3, 0.6, 1.25, 2.5, 5.0 and 10.0 μg/ml of $M_5$32. (C) Graph derived from quantitation of assays result by phosphorimager showing a dose–response for inhibition of 3′-joning (strand transfer) of HIV-1 integrase produced by $M_5$22 and $M_5$32 drugs.

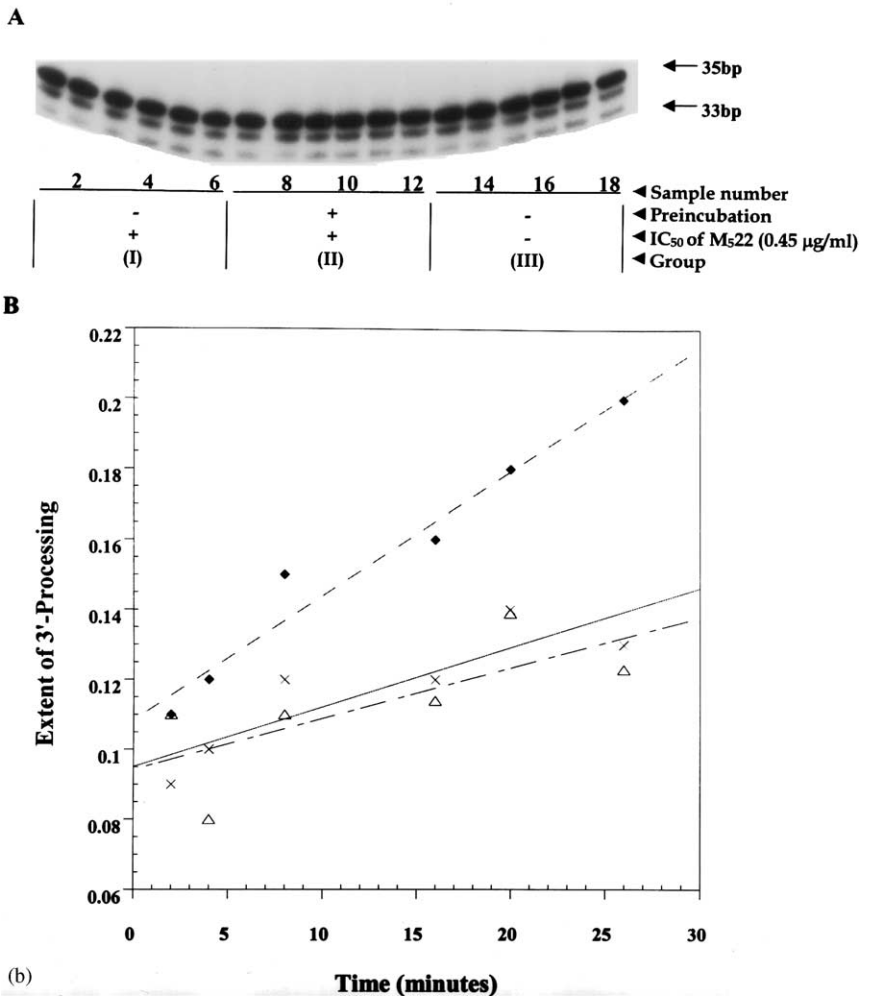


Fig. 7. Kinetic and mechanistic inhibition of HIV-1 integrase by $M_5$22. (A) Formation of the 3′-processing product (33 bp) from the DNA substrate (35 bp) at incubation times of 2, 4, 8, 16, 20 and 26 min. Group I, lanes 1–6, no pre-incubation of drug with HIV-1 integrase. Group II, lanes 7–12, HIV-1 integrase pre-incubated with drug. Group III, no drug and no pre-incubation period prior to the reaction. (B) Quantitation of results in (A) by phosphorimaging analysis, -×-, $M_5$22 without preincubation; -△-, $M_5$22 with preincubation; and -■-, no drug and no preincubation.

tion (Hase et al., 1997), improvement of uremic symptoms (Tanaka et al., 1989), cause endothelium-dependent vasodilation in the aorta (Kamata et al., 1993) and correction of hypertension (Kamata et al., 1994). In this communication, we report the potent anti-HIV activity of pure compounds, lithospermic acid and lithospermic acid B for the first time. These two isolated pure compounds were found to suppress HIV production by inhibition of the viral integration process with an exceedingly high therapeutic window.

In a review of the integrase inhibitors, Pommier et al., (2000) have made an extensive examination of currently available retroviral integrase inhibitors and have listed them according to their drug targeting sites. The list of integrase inhibitors includes; diketo acids, L-731, 988 and L-708,906 (Hazuda et al., 2000) and 5 CITEP (Goldgur et al., 1999), tricyclic nucleotides (Zhang et al., 1998), pisopdApdC (Taktakishivli et al., 2000), guanosine quartets (Jing et al., 2000), thiazolothiazepines (Neamati et al., 1999), chicoric acid

derivatives specifically caffeoylquinic acids (Neamati et al., 1997a,b; Robinson, 1998; King and Robinson, 1998; King et al., 1999; Lin et al., 1999; Zhu et al., 1999), integric acid derivatives (Singh et al., 1999), equisetin and phomasetin (Hazuda et al., 1999; Singh et al., 1999), lamellarin α-20-sulfate (Reddy et al., 1999), salicylhydrazides (Neamati et al., 1998), polyhydroxylated styrylquinolines (Mekouar et al., 1998; Zouhiri et al., 2000) and members of ribosome inactivating proteins such as MAP30, luffin and saporin (Wang et al., 1999; Au et al., 2000). The target sites for these compounds have been mapped by a variety of binding studies, primarily using recombinant wild type and mutant HIV integrase. The efficacies of these integrase inhibitors have also been evaluated by studying their effect on integrase activities (3′-processing and strand transfer) and integration in vitro, their effect on viral replication in vivo and in some cases their toxicity in cell culture. An effective dose range for inhibition in vitro integrase activity has been reported for these compounds. Most of them were also found to be somewhat effective in suppressing HIV in vivo, but required even higher concentrations than those reported for inhibition of integrase activities in vitro. Cellular toxicity at these higher drug concentrations was observed for many of these integrase inhibitors, a point of major concern for investigators.

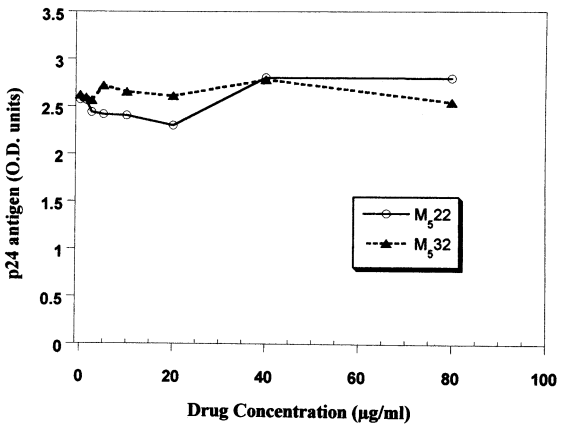


Fig. 8. Effect of $M_5$22 and $M_5$32 on HIV-1 entry in H9 cells. The cells were incubated for 1 h with HIV-1_{MN} in the presence of various concentrations, 0, 1.25, 2.5, 5, 10, 20, 40 and 80 μg/ml of $M_5$22 and $M_5$32. P24 antigen bound to H9 cells was determined for both drugs using HIV/p24 monoclonal antibody assay.

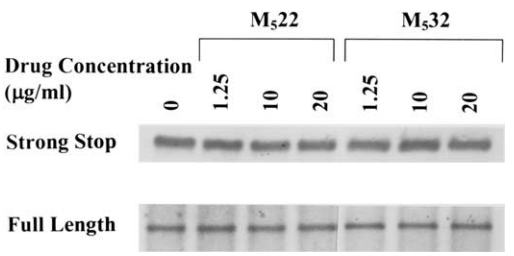


Fig. 9. Effect of $M_5$22 and $M_5$32 on strong stop and full-length of HIV-1 DNA in infected H9 cells. Synthesis of strong and full-length viral DNA in H9 cells infected with HIV-1_{MN} in the presence of various concentrations, 0, 1.25, 10, 20 μg/ml of $M_5$22 and $M_5$32.

Among all the integrase inhibitors from this year 2000 list (Pommier et al., 2000), compounds L-731, 988 and L-708, 906, the two diketo acids, have shown as two exceptionally promising integrase drugs. Both compounds inhibit pre-integration complex (PIC)—targeted integration in vitro (IC_{50} of 80 and 50 nM, respectively). They suppress HIV-1 replication effectively with an IC_{50} of 1–2 μM. The antiviral activity of these compounds is due exclusively to inhibition of one of the two catalytic functions of integrase, strand transfer (Hazuda et al., 2000).

Compared to the two diketo acids, our inhibitors, lithospermic acid ($M_5$22) and lithospermic acid B ($M_5$32), showed comparable IC_{50} values in suppressing HIV replication in H9 cells (IC_{50} of 2 and 6.9 μM for $M_5$22 and $M_5$32, respectively). In addition, $M_5$32 and $M_5$22 were found to be non-toxic to H9 cells in culture ($CC_{100} > 223$ μM for $M_5$32 and 297 μM for $M_5$22). Furthermore, our inhibitors showed lower IC_{50} values (0.48 μM for $M_5$32 and 0.84 μM for $M_5$22) than the IC_{50} reported for the diketo acids (6 μM) in inhibiting the 3′-processing of HIV-1 integrase activity. In recent studies (Singh et al., 2001), they reported that isocomplestatin inhibited HIV-1 integrase strand transfer activities with IC_{50} of 4 μM and in another studies using nuclease-resistant, non-natural dinucleotide inhibitor for HIV-1 integrase, they showed that 3′-processing was inhibited with the IC_{50} of 7.5 μM and strand transfer with IC_{50} of 5.9 μM (Taktakishivli et al., 2001). By comparing our inhibitors, $M_5$22 and $M_5$32, have much lower IC_{50}

of 0.48 and 0.37 μM, respectively for inhibiting strand transfer activities of HIV-1 integrase. These low IC_{50} values for inhibition of HIV-1 replication, HIV-1 integrase activities for 3′-processing and strand transfer seem to suggest that $M_5$22 and $M_5$32 are potent and can likely be useful as therapeutic drugs for AIDS. In addition, both $M_5$22 and $M_5$32 are highly specific in inhibition of HIV-1 integration and they are small water soluble molecules. They do not prevent HIV entry, show no inhibition of reverse transcriptase activity since the levels of intracellular strong stop and full-length viral DNA remained unchanged in infected H9 cells following drug treatment. $M_5$22 and $M_5$32 inhibited the catalytic activities of HIV-1 integrase, and 3′-joining to the target DNA in the presence of exceedingly low concentrations of drugs. Kinetic and mechanistic studies of $M_5$22 inhibitor further suggested that it binds to HIV-1 enzyme and inhibited enzymatic reaction at a fast rate. $M_5$22 and $M_5$32 are, therefore, selective integrase inhibitors. Their potency against HIV without cellular toxicities: make them extremely appealing as anti-HIV testing agents in vivo.

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