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The anti-HIV-1 effect of scutellarin

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Abstract

Scutellarin was purified from the plant *Erigeron breviscapus* (Vant.) Hand.–Mazz. The activity against 3 strains of human immunodeficiency virus (HIV) was determined in vitro in this study. These were laboratory-derived virus (HIV-1_{IIIB}), drug-resistant virus (HIV-1_{74V}), and low-passage clinical isolated virus (HIV-1_{KM018}). From syncytia inhibition study, the EC_{50} of scutellarin against HIV-1_{IIIB} direct infection in C8166 cells was 26 μM with a therapeutic index of 36. When the mode of infection changed from acute infection to cell-to-cell infection, this compound became even more potent and the EC_{50} reduced to 15 μM. This suggested that cell fusion might be affected by this compound. By comparing the inhibitory effects on p24 antigen, scutellarin was also found to be active against HIV-1_{74V} (EC_{50} 253 μM) and HIV-1_{KM018} (EC_{50} 136 μM) infection with significant difference in potency. The mechanism of its action was also explored in this study. At a concentration of 433 μM, scutellarin inhibited 48% of the cell free recombinant HIV-1 RT activity. It also caused 82% inhibition of HIV-1 particle attachment and 45% inhibition of fusion at the concentrations of 54 μM. In summary, scutellarin was found to inhibit several strains of HIV-1 replication with different potencies. It appeared to inhibit HIV-1 RT activity, HIV-1 particle attachment and cell fusion. These are essential activities for viral transmission and replication.

Keywords: Scutellarin; HIV-1; Anti-HIV agent; Reverse transcriptase; HIV entry

Conventional combination therapy with reverse transcriptase (RT) and protease inhibitors greatly reduce morbidity and mortality in HIV-1 infected individuals. However, use of these drugs was limited by their many side effects and development of drug-resistant. Continuous development of new anti-HIV-1 drugs appeared to be inevitable. In recent years, a variety of compounds were discovered as having anti-HIV-1 action. These compounds acted on various processes of the viral life cycle like adsorption, entry, fusion, integration, and maturation. Some even targeted at more than one process [1–3]. For example, prostratin interfered with viral entry or virus-cell adsorption/fusion. It offered great potential therapeutic use on HIV-1 infections [4]. Several plant flavonoids, such as baicalein, quercetin, had been shown to inhibit HIV-1 RT activity [5,6].

Erigeron breviscapus (Vant.) Hand.–Mazz. is used as traditional herbal medicine in China and scutellarin is a polyphenolic flavonoid purified from this plant. It is being used in the treatment of cerebral infarction, infantile acute viral myocarditis, and pulmonary heart disease [7–9]. The pharmacokinetics of scutellarin in dogs and rabbits had been described [10,11]. The present study examined its anti-HIV-1 action and possible mechanism of action.

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Materials and methods

Chemicals and reagents. Scutellarin ($C_{21}H_{18}NO_{12}$) with a molecular weight of 462 was extracted from *E. breviscapus* (Vant.) Hand.–Mazz. Air-dried whole plant of *E. breviscapus* (500 g) was extracted with 80% ethanol under reflux for 3 h. The combined organic phase was dried under vacuum to yield 16 g residue. It was then further purified by silica gel (320 g, 200–300 mesh) chromatography with a mixture of solvents containing $CH_3Cl/MeOH/H_2O$ (8:2:0.2—6.5:3.5:0.5, v/v). The crude scutellarin obtained was crystallized in ethanol to yield about 2.6 g of pure scutellarin. Purity of scutellarin determined by HPLC was over 99%. Scutellarin was dissolved in complete medium for all assays carried out in this study.

3′-Azido-3′-deoxythymidine (AZT) and dextran sulfate (DS) were purchased from Sigma. Horseradish peroxidase (HRP)-labeled goat anti-human IgG was purchased from Sino-America Biotechnology (China). Monoclonal antibody (McAb) to HIV-1 p24 was produced in our laboratory. Human polyclonal anti-HIV-1 antibody was kindly donated by Dr. H Hoshino (Gunma University School of Medicine, Japan). T-20 (enfuvirtide) and colorimetric RT assay kits were purchased from Roche Molecular Biochemicals.

Cells and viruses. Cell lines used in this study (H9, C8166, MT-2, and H9 chronically infected with HIV-1_{IIIB}) were maintained in RPMI-1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco). The cells used in all experiments were in log-phase growth. PBMC from healthy donors were isolated by Ficoll–Hypaque centrifugation and incubated in complete medium containing 5 μg/ml phytohemagglutinin (PHA) (Sigma) for 72 h prior to use for antiviral assays. The laboratory-derived viruses HIV-1_{IIIB} and the drug-resistant virus HIV-1_{74V} were obtained from NIH, AIDS Reagent program and MRC, AIDS Reagent Project, UK. The clinically isolated HIV-1_{KM018} was obtained from a HIV-1 infected individual of Yunnan Province as described [12]. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) was determined and calculated by Reed and Muench method. All the viruses were stored in small aliquots at −70 °C.

Cytotoxicity assay. Cytotoxicity was measured by MTT method as described previously [13]. Briefly, cells were seeded in the absence or presence of various concentrations of scutellarin in triplicate for 3–7 days. The percentage of viable cells was quantified at 595/630 nm ($A_{595/630}$) in an ELISA reader (Elx800, Bio-Tek Instrument, USA). The cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was determined from dose–response curve.

Syncytium reduction assay. Different concentrations of scutellarin were added in a 96-well microtitre plate. C8166 or MT-2 (3×10^4 cells/well) were seeded and inoculated with 100 $TCID_{50}$ HIV-1 and then incubated at 37 °C in a humidified incubator with 5% CO_2 for a period of 72 h. Control assays were performed without the testing compounds in HIV-1-infected and uninfected cultures. AZT was used for drug control. The number of syncytium (multinucleated giant cell) in each well was counted under an inverted microscope [14]. Percentage inhibition of syncytial cell formation was estimated from the percentage of syncytial cell number in treated culture to that in infected control culture.

Inhibition of HIV-1 p24 antigen production in acute infection. The effect of scutellarin on HIV-1 replication in vitro was also measured by p24 expression using capture ELISA as described previously [15]. Briefly, MT-2 or C8166 cells were inoculated with HIV-1_{74V} or HIV-1_{IIIB} at an MOI of 0.03, respectively, at 37 °C for 2 h to allow for viral absorption. It was then washed three times with PBS. The cells were plated at 3×10^4/well with or without the addition of scutellarin. HIV-1 p24 expression was assayed in cell-free supernatants harvested at day 4.

Inhibition of HIV-1 p24 antigen production in chronically infected cell lines. H9 cells chronically infected with HIV-1_{IIIB} were washed three times with PBS to remove free virus particle. 200 μl/well (3×10^5 cell/ml) of the cell suspension was cultured for 3 days in a 96-well culture plate with different concentrations of scutellarin. Three wells without scutellarin were used as negative control. After 3 days of incubation, p24 antigen in the culture supernatants was tested by ELISA.

RT assay. HIV-1 RT activity was measured by ELISA RT kit using a commercially available kit according to the protocol provided by the manufacturer. Samples were incubated with DIG-labeled-reaction mixture at 37 °C for 15 h. Anti-DIG-POD solution was added afterward followed by substrate ABTS. The absorbance at 405/490 nm ($A_{405/490}$) was determined in the ELISA reader [16].

Inhibition of HIV-1 p24 antigen production in PBMC. Adequate numbers of PHA-activated normal PBMC were inoculated with HIV-1_{KM018} (MOI = 0.03). After 2 h of virus adsorption, the cells were washed twice with PBS and incubated with or without scutellarin in culture medium supplemented with 50 U/ml human recombinant IL-2 at 1×10^6 cells/ml for 7 days. Half of the medium was changed twice per week with corresponding scutellarin concentrations. At 7 days post-infection, HIV-1 p24 antigen in the culture supernatants was analyzed by ELISA. The inhibition of HIV-1 p24 antigen production in PBMC was calculated.

Assay of HIV-1 particle attachment and entry. HIV-1 entry was estimated from the concentration of intracellular virus RNA by real-time RT-PCR. C8166 cells were pretreated with different concentrations of scutellarin for 1 h. It was then inoculated with HIV-1_{IIIB} and allowed to adsorb with virus for 2 h at 37 °C. HIV-1 bound on the cell surface was removed by trypsinization, and then washed three times with PBS. The attachment of HIV-1 to cells was monitored after 1 h of incubation with HIV-1_{IIIB} at 4 °C, and then washed extensively with PBS to eliminate unbound HIV-1 particles [17,18]. T20 and DS were used as control. The amount of RNA in cell extracts was measured by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

HIV-1 RNA was amplified with a commercial HIV-1 PCR fluorogence diagnostic kit (PG Biotech). Total RNA from cultured cells was reverse-transcribed into cDNA. Amplification of specific PCR products was detected by SYBR Green real-time PCR. The PCR cycling conditions were 1 cycle at 42 °C for 30 min and 95 °C for 3 min, 5 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and then followed by 40 cycles of 5 s at 95 °C, 30 s at 60 °C, on an GeneAmp 5700 Sequence Detection System (PE Applied Biosystems).

Cell fusion assay. C8166 cells (2×10^5) were pretreated for 1 h with varying concentrations of scutellarin prior to mixing with 3×10^4 HIV-1_{IIIB} chronically infected H9 cells in 96-well plate. After co-culture for 6 h at 37 °C in 5% CO_2, syncytia formation was scored through an inverted microscope.

Results

Anti-HIV-1 action of scutellarin

The molecular structure of scutellarin is well defined and shown in Fig. 1. The antiviral actions of this compound on 3 strains of HIV-1 were summarized in Table 1. Scutellarin significantly inhibited syncytia formation in C8166 induced directly by HIV-1_{IIIB} in a

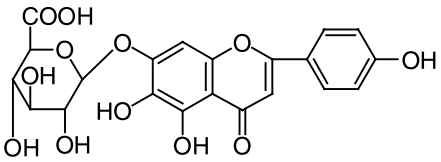

Fig. 1. Structural formula of scutellarin.

Table 1
The summary of cytotoxicity and anti-HIV-1 activities of scutellarin

Cells	Strains	Assays	EC_{50} (μM)	CC_{50} (μM)
C8166	HIV-1_{IIIB}	Syncytia /MTT	26	945
		p24/MTT	175	945
MT-2	HIV-1_{74V}	p24/MTT	253	>1082
PBMC	HIV-1_{KM018}	p24/MTT	136	336
C8166	HIV-1_{IIIB}/H9	Cocultivation	15	ND

EC_{50} is the effective concentration that inhibits 50% of viral production, CC_{50} is the inhibitory concentration that reduces cellular growth or viability of uninfected cells by 50%. The data shown in the table are a representative of three independent experiments. ND, not determined.

dose-dependent manner with an EC_{50} value of 26 μM (Table 1 and Fig. 2). This demonstrated that scutellarin inhibited manifestation of viral infection. When the mode of infection changed from an acute infection to a cell-to-cell infection, scutellarin became an even more potent inhibitor. This indicated that viral attachment or cell fusion maybe interfered by scutellarin.

Viral proliferation was assessed by measuring the HIV-1 p24 antigen. Results showed that scutellarin inhibited acute HIV-1_{IIIB} infection on C8166 cells with EC_{50} value of 175 μM (Table 1 and Fig. 3). It was less potent against the resistant strain HIV-1_{74V} in the MT-2 cell with a mean EC_{50} of 253 μM and more potent against the low-passage clinical isolated virus (HIV-1_{KM018}) with EC_{50} of 136 μM (Table 1 and Fig. 3). Scutellarin was totally ineffective against H9 cells chronically infected with HIV-1_{IIIB} (Fig. 3).

Cell viability

Cytotoxicity was measured in parallel with the determination of antiviral activity. The effect of scutellarin on the viability of C8166, MT-2, and PBMC cells was examined (Fig. 4). Results indicated that the CC_{50} of scutellarin for the cells ranged from 336 to >1082 μM (Table 1).

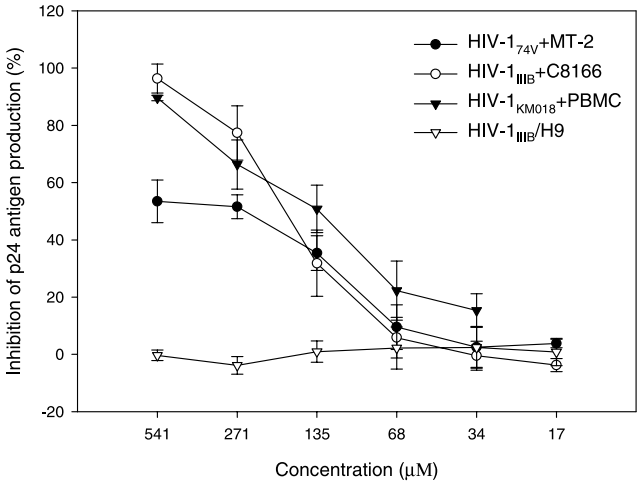

Fig. 3. The effect of scutellarin on different strains of HIV-1 replication measured by p24 antigen production. Inhibition of HIV-1_{74V} in acutely infected MT-2 cell (●), inhibition of HIV-1_{IIIB} in acutely infected C8166 cell (○), inhibition of HIV-1_{KM018} in acutely infected PBMC (▼), and the effect on chronically infected HIV-1_{IIIB}/H9 (▽). Data are expressed as means ± standard deviations.

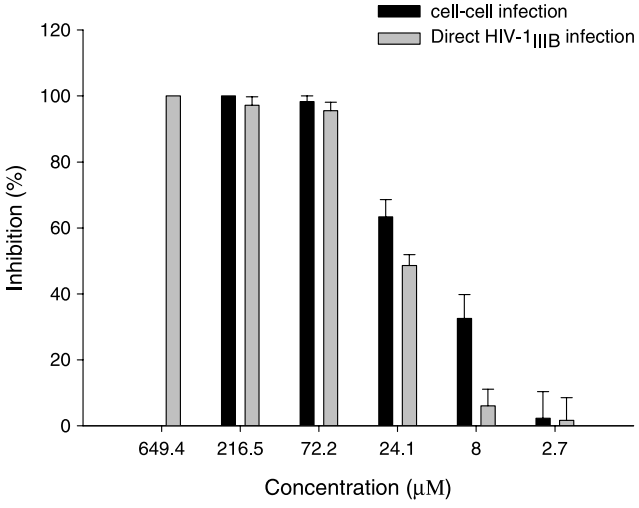

Fig. 2. The effect of scutellarin on HIV-1_{IIIB} replication measured by syncytia formation. Data are expressed as means ± standard deviations.

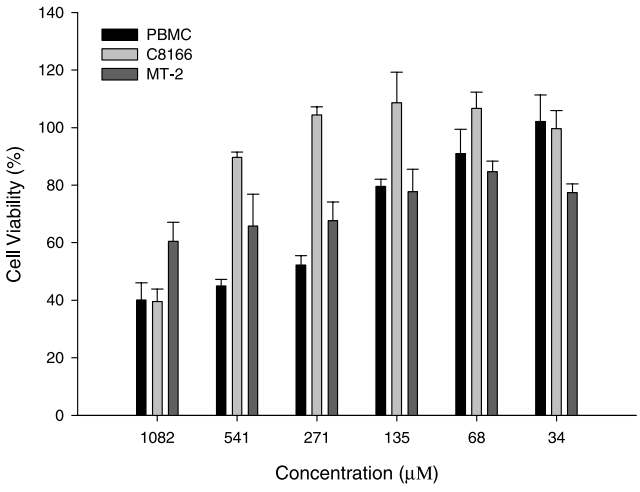

Fig. 4. In vitro cytotoxicity of scutellarin in various cells. Data are expressed as means ± standard deviations.

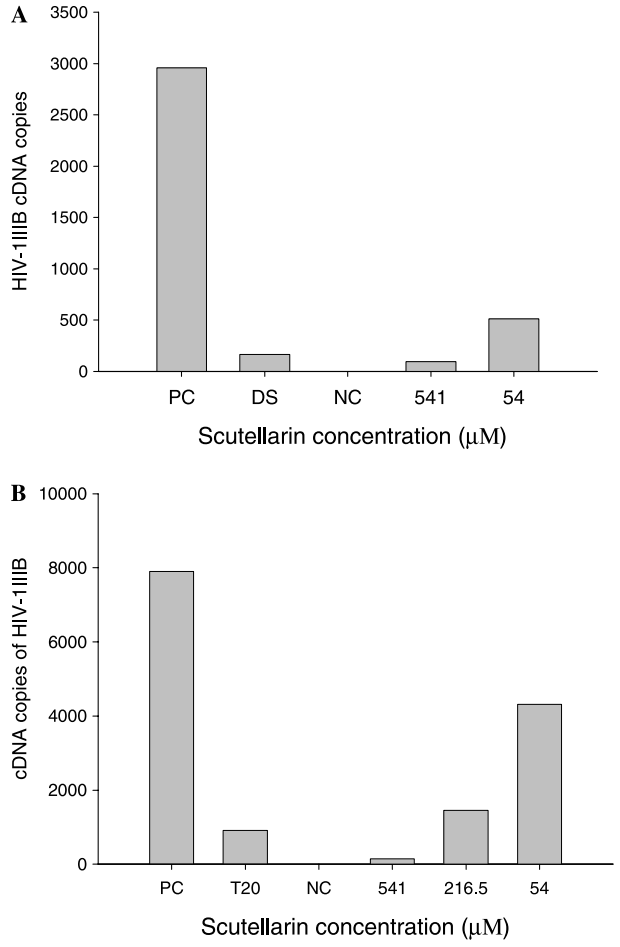

Fig. 5. Inhibition of HIV-1 attachment and entry. Experimental conditions for HIV-1 attachment (A) and entry (B) as described in materials and methods. DS 250 μg/ml or T20 1 μg/ml as a reference of anti-HIV-1 agent, positive control (PC) was provided by addition of compound-free medium, normal cells were used as a negative control (NC). Data are a representative of three independent experiments, each performed in duplicate.

Mechanism of action

Mechanistic evaluation demonstrated that scutellarin inhibited the enzymatic activity of purified recombinant HIV-1 RT. Scutellarin inhibited 48% of the RT activity of at a concentration of 433 μM. To explore if the effect on HIV-1 replication was caused by the inhibition of viral entry, we also analyzed the capacity of scutellarin to inhibit HIV attachment and entry. Results showed that scutellarin caused 82% inhibition of HIV-1 particle attachment (Fig. 5A) and 45% inhibition of fusion (Fig. 5B) at the concentrations of 54 μM.

Discussion

At present, most FDA approved anti-HIV-1 drugs are aimed at blocking HIV-1 replication by inhibiting either HIV-1 RT or protease enzymes. Alternative targets of inhibition are always necessary. Virus entry is an attractive target for the development of new anti-HIV-1 agents. In recent years, considerable advances have been made in identifying agents that inhibit HIV-1 entry into cells. Clinical trials on some of these compounds like PRO 542, SCH-C, and AMD 3100 were done [19,20]. Recently, a third family of antiviral drug-fusion inhibitor T-20 (enfuvirtide) has been approved by the US FDA. The discovery of T-20 generated hope for identifying inhibitors that target at early steps of the HIV-1 life cycle. The development of entry inhibitors will greatly expand the therapeutic options for treatment of HIV-1, particularly for patients who harbor multi-drug-resistant viruses.

In the present study, it was found that scutellarin processed anti-HIV-1 activities in several strains of HIV-1 (summarized in Table 1). These included a laboratory-derived virus ($HIV\text{-}1_{IIIB}$), drug-resistant virus ($HIV\text{-}1_{74V}$), and low-passage clinical isolated virus ($HIV\text{-}1_{KM018}$). The potency and toxicity have variations that may be due to difference in cell type or a reflection of the preferential actions of scutellarin on these cells.

There were direct evidence that scutellarin interfere with the entry of HIV-1 particles into cells (Fig. 5). This is supported by the observation that scutellarin is more potent in cell-to-cell infection than direct viral infection. A lack of inhibitory activity against chronically infected H9 also suggests that the mechanism of action of scutellarin was at an early step.

Scutellarin is one of the flavonoides used clinically to treat cerebral vascular patients in China. There are large numbers of natural compounds belonging to flavonoides family. Some of them exhibit anti-HIV-1 effect in vitro and often with more than one mode of actions. They can interact at different steps in the life cycle of HIV-1, including viral entry [21–23], RT, integrase [24,25], and Vpr [26]. Many anti-HIV-1 agents (e.g., suramine, michellamine, and glycyrrhizin) also showed significant PKC inhibition [27–29]. Scutellarin also has an inhibitory effect on PKC [30,31] and therefore PKC inhibition may be one of the mechanisms by which scutellarin inhibits HIV-1 replication. It is premature at this stage to define the mechanism of action of scutellarin. There were some evidences that scutellarin interfere with viral attachment, cell fusion, RT inhibition, and PKC inhibition. The anti-HIV-1 action of scutellarin may be related to one or more of these activities.

In conclusion, scutellarin was shown to inhibit HIV-1 replication. This action may be related to cell entry and/or RT inhibition.

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