Extract of *Prunella vulgaris* spikes inhibits HIV replication at reverse transcription *in vitro* and can be absorbed from intestine *in vivo*

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It has been reported that extracts of the spike of *Prunella vulgaris* (PS) exhibit anti-HIV activity at the adsorption and reverse transcription stages. In this study, the actual activity of PS in cells, kinetic analysis of the inhibitory activity of PS against HIV reverse transcriptase and the feasibility of oral administration were examined. First, to clarify whether this extract shows anti-HIV activity in cells *in vitro*, the number of copies of proviral DNA in HIV-exposed cells was calculated. The number of copies was significantly decreased in cells cultured in the presence of PS extract, but not in the presence of dextran sulphate. The activity of PS extract in the cells was also assessed by the drug addition test, during and after HIV adsorption. PS extract and dextran sulphate suppressed HIV production to similar levels when added after HIV adsorption. However, only PS extract suppressed HIV production at the same concentration when the drugs were added during HIV adsorption. Presumably, the penetration of the PS extract into the cells was required for this activity. Secondly, fractionated PS inhibited HIV reverse transcription in a non-competitive manner. This fractionated PS kept anti-HIV activity, but inhibited HIV replication and adsorption to a lesser extent compared to dextran sulphate. Lastly, an active component(s) was detected in plasma *in vivo*, after injection into the intestine, which demonstrates the feasibility of oral administration dosing.

Keywords: *Prunella vulgaris*; Prunella spike; HIV; reverse transcriptase; intestinal absorption.

Introduction

Over the last decade, several anti-human immunodeficiency virus (HIV) drugs have emerged in the clinical setting, such as nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors, and protease inhibitors. Many studies provide a positive message supporting the use of potent combination antiretroviral regimens. Use of this therapy has resulted in remarkable declines in hospitalization rates, morbidity and mortality, where the drugs are available (Hogg *et al.*, 1997, 1998; Palella *et al.*, 1998). However, choices for combination regimens are not a simple reflection of possible permutations derived from a list of available drugs. Practical issues such as drug compatibility, adverse effects and cross-resistance limit the options available, especially when there is drug failure and resistance (Carpentier *et al.*, 1998). There is therefore still a demand to develop newly discovered drugs. Medicinal plants may be the source of drugs in the future, or may be important in alternative therapeutic approaches in developing countries, at the center of the AIDS pandemic. Undoubtedly, currently available drug regimens are not readily available in these countries for economic reasons.

Extracts from the spike of *Prunella vulgaris* have previously been examined for anti-HIV activity, both as crude extract, and as partially purified material (Chang & Young, 1988). One of the purified components of this extract, prunellin, is identified as a sulphated polysaccharide that, by gel permeation chromatography and the Squire method on Sephadex G-75, was found to be approximately 10 kDa in size (Ishiba *et al.*, 1989). The extract is able to inhibit significantly HIV-1 replication with relatively low cytopathicity. Preincubation of HIV-1 with the purified extract dramatically decreases its infectivity. This implies that the extract inhibits the binding of HIV to the surface of susceptible cells (Yao *et al.*, 1992). Yamashiki and colleagues have reported that the extract inhibits the activity of HIV reverse transcriptase *in vitro* (Yamashiki *et al.*, 1998).

With a view to the clinical application of this extract, the exact mode of action, a kinetic analysis of the inhibitory activity against HIV RTase, and the possible absorption of this extract through the intestine *in vivo* were investigated in this study.
Materials and Methods

Prunella spike extract and other drugs
Prunella spike (PS), the dried material of the spike of Prunella vulgaris, which is listed in the Japanese Pharmacopoeia, was purchased from Toda-shoken in Thailand (Osaka, Japan). (The term PS has been used in this manuscript after its designation in the Japanese Pharmacopoeia.) The dried material was boiled under reflux for 1 h. The extract was then dried again and finally, the dried material was reconstituted with water and used for the experiments described below. Maximum concentration of this preparation was 20 mg/ml. The control drugs, of 2',3'-dideoxyinosine (didanosine) and dextran sulphate (molecular weight 8000) were purchased from Sigma-Aldrich.

Fractionation of PS
Hot water extract of PS was mixed with ethyl acetate. The anti-HIV activity remained only in the water layer. The materials remaining in the water layer were subsequently subjected to analysis by HPLC using a Shim-pack CLC-ODS(M) column (Shimazu, Kyoto, Japan). This column comprises 4.6 mm ID×25 cm stainless steel tubes packed with totally porous, spherical silica particles (5 μm particle diameter, 100 Å pore diameter) with chemically modified surfaces. The gradient curve was made to mix acetonitrile with 10 mM sodium phosphate. The ratio of acetonitrile was increased from 0 to 10% in the initial 5 min, and from 10 to 80% in the next 13 min. A single peak was detected at 254 nm in the second gradient. This fraction was collected, evaporated, dried and reconstituted with DMSO.

Cells and viruses
Human T cell lymphotropic virus type 1-immortalized T cell lines MT-2 and MT-4 cells were used as the target cells to assess the anti-HIV activity after exposure to HIV-1_LAI in the absence or presence of drugs. HIV-1_LAI had been propagated in MT-4 cells and stored at −80°C prior to use. A monoclonal cell line, U937, was also infected with HIV-1_LAI and cultured for 6 months. All the U937 cells were determined to be infected by flow cytometry.

HIV-induced CPE and drug-induced cytotoxicity
MT-2 and MT-4 cells were exposed to HIV-1_LAI for 1 h and cultured in 1 ml of fresh culture medium consisting of RPMI-1640 supplemented with 0.03% L-glutamine, 0.2% sodium bicarbonate, 0.006% Kanamycin sulphate, 10% undialysed and heat-inactivated fetal bovine serum. Culture was initiated to adjust the number of cells to 2×10^5 cells/ml in the absence or presence of drugs. On day 4 after infection, the number of viable cells was calculated by the Trypan blue exclusion method. Drug-induced cytotoxicity was also assessed simultaneously by the number of viable cells not exposed to HIV and cultured in the presence of drugs. The effect of a drug on cell survival was measured from the percentage of surviving cells exposed to HIV-1 in the presence of drug, using the following formula (Kageyama et al., 1992):

\[ \frac{(N_1-N_2)\times(N_3-N_4)}{(N_2-N_4)\times100} \]

where \( N_1 \) is the number of cells exposed to HIV-1 and cultured in a certain concentration of drug, \( N_2 \) is the number of HIV-1-exposed cells in the absence of drug, and \( N_3 \) is the number of cells cultured in the absence of drug and HIV exposure. IC_{50} and IC_{90} values were inferred from the concentrations that allow the survival of 50 and 90% of HIV-exposed cells, respectively.

Quantification of HIV-1 production
The production of HIV-1 in the culture medium was assessed by measuring the concentration of HIV-1 Gag p17 by ELISA (kindly provided by K Taniguchi). Recombinant p17 was used to draw the standard curve (Taniguchi et al., 1998).

Adsorption inhibition test by morphological change
MT-2 cells (2.5×10^6) and HIV-1_LAI-infected U937 cells (2.5×10^6) were pretreated with PS at 50 μg/ml for 4 h. The two cell lines were then mixed and cocultured for 3 h before being observed for morphological changes such as giant cell formation under a light microscope.

Adsorption inhibition test
To assess the antiviral activity at adsorption of the extract of PS and PS fraction by HPLC, dextran sulphate and didanosine were used as the control (Baba et al., 1988; Mitsu et al., 1988; Hartman et al., 1990; Mitsu & Broder, 1986). Inhibition of adsorption by PS extract was assessed by adding the drug during HIV adsorption for 2 h. Briefly, MT-4 cells were incubated with HIV-1_LAI at 0.04 TCID_{50} per cell, for 2 h, in the presence of various concentrations of the extract of PS, didanosine, and dextran sulphate. Cells were then washed extensively and cultured for 4 days. In contrast, drugs were added after HIV adsorption in the next experiment. Briefly, cells were incubated with HIV-1_LAI in the absence of drug for 2 h, then extensively washed and cultured in the presence of the drug for 4 days. The number of viable cells and the amount of Gag produced on day 4 in culture were assessed as described above. The anti-HIV activity of fractionated PS at adsorption was also assessed. This time cells were pretreated, and challenged by HIV afterwards. Briefly, MT-4 cells were pretreated with drugs at IC_{50} and IC_{90} for 2 h, washed extensively, then challenged with HIV-1. Subsequent culture was performed in the absence of drugs for 4 days. IC_{50} and IC_{90} were 5 and 20 μM for didanosine,
0.5 and 0.8 μM for dextran sulphate (Figure 1). Those values for the fractionated PS corresponded to 500- and 200-fold diluted fractionated materials, respectively. The number of viable cells on day 4 and Gag produced in culture medium during the culture period were measured by the methods described above.

Competitive DNA PCR
MT-2 cells were incubated with HIV-1_LAI at 0.007 TCID$_{50}$ per cell for 1 h, and cultured for the remaining 18 h in the absence or presence of PS (50 μg/ml), didanosine (20 μM), and dextran sulphate (0.8 μM). These cells were suspended in solution (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA), adjusted to contain 0.6 mg/ml of proteinase K and 0.3% SDS and incubated at 37°C for 1 h. After repeated extraction with phenol:chloroform:isoamyl alcohol (25:24:1), sample DNA was precipitated with ethanol. Prepared DNA (0.15 μg) was subjected to PCR with competitor DNA, serially diluted and adjusted at appropriate concentrations as reported elsewhere (Menzo et al., 1992; Piatak et al., 1993). The primers MZ8 (5'-GCATTATCAGAAGGAGCCAC-3', 1316-1335 of ARV2) and MZ9(AGGGTACTAGTGTCTTGCGC-3', 1524-1505) had been reported previously (Zeevi et al., 1992), and were designed to amplify an internal fragment of either 209 bp (from wild-type HIV-1 target sequences) or 189 bp (from the pMZAL competitive template). A plasmid (pMZAL) containing a 20 bp internal deletion was prepared. Amplification was performed with 30 cycles (94°C for 1 min, 50°C for 1 min and 72°C for 1 min), followed by a final incubation at 72°C for 5 min. After amplification, 20% of each product was subjected to electrophoresis using a Spreadex XL 400 mini gel (Gietz Elchrom Scientific, Switzerland). The density of areas (WA, wild area; DA, deleted area) of both amplified products was calculated by Densitograph version 4.0 (Atto, Tokyo, Japan). DA was corrected (DAc) for its molar ethidium bromide incorporation as reported elsewhere (Menzo et al., 1992) as follows: $DAc = DA \times (\text{wild-type length} / \text{deleted length}) = DA \times 1.11$. The DAc/WA ratio was calculated for each sample, and plotted against the copy number of the deleted competitor. A single regression curve was fitted for the positive controls and for each sample. The copy number of the wild-type template could be calculated from the curve expression, 'DAc/WA = 1'.

HIV-1 RT activity
The inhibitory activity of fractionated PS against HIV RT was measured to add 10 μl of this sample to a final volume of 100 μl reaction mixture, containing 40 mM Tris-HCl pH 8.0, 40 mM KCl, 4 mM DTT, 10 mM MgCl$_2$, 20 units/ml HIV-1 RT (Seikagaku Corp., Tokyo, Japan), 20 μCi/ml (1 μM) [3H]-thymidine triphosphate (TTP), and 50 μg/ml poly(rA), p(dT)$_{12-18}$. After the incubation of reaction mixture at 37°C for 5 min, 25 μl of the reaction mixture was spotted onto a filter (Type 2, Toyo Roshi Kaisha Ltd, Tokyo, Japan). The filter was then soaked in 10% trichloroacetic acid (TCA) in 0.02 M pyrophosphate, and subsequently washed twice with 5% TCA, before the radioactivity in the product formed was counted (Poiesz et al., 1980; Unno et al., 1995). The $K_d$ value was determined from initial linear steady-state velocities using
Figure 2. Inhibition of HIV replication at adsorption determined by morphological change

MT-2 cells or HIV-infected U937 cells were pretreated with PS extract at 50 μg/ml for 48 h. These were then mixed together, cultured and observed for morphological change such as giant cell formation, under a light microscope. In the absence of drug, giant cells and balloon-shaped cytoplasm were formed and could be easily visualized under a light microscope. By either pretreatment of uninfected cells or HIV-1 infected cells with PS extract, morphologically altered cells were not recognized after mixing of these cells.

Figure 3. Inhibition of proviral DNA synthesis

<table>
<thead>
<tr>
<th>Time</th>
<th>No drug</th>
<th>PS</th>
<th>Didanosine</th>
<th>Dextran sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000*</td>
<td>10^6</td>
<td>10^5</td>
<td>1000</td>
<td>10^4</td>
</tr>
<tr>
<td>200**</td>
<td>&gt;670 (&gt;420)</td>
<td>100 (0)</td>
<td>150 (0)</td>
<td>650 (400)</td>
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<tr>
<td>300</td>
<td>&gt;670 (&gt;420)</td>
<td>410 (10)</td>
<td>260 (30)</td>
<td>&gt;670 (&gt;420)</td>
</tr>
</tbody>
</table>

PCR products of HIV provirus and plasmid (pMZ4A) containing a 20 bp internal deletion were designated as 'sample' and 'standard', respectively. The mean value at time 0 was subtracted from each value (shown in parentheses). *The number of copies of competitor in reaction mixture. **x1000 copies per μg of total DNA in the cells.

Lineweaver–Burke plot analysis to draw the linear lines according to the simple curve fit equation of Cricket graph (version 1.2; Cricket Software, Pa., USA). The apparent K_i value was determined graphically by measuring velocities in the presence and absence of fixed concentrations of fractionated PS (Dixon & Webb, 1979).

Absorption test from intestine
Under chloral hydrate anesthesia at 300 mg/kg, we made an abdominal longitudinal incision of skin and muscles in guinea pigs to observe an intestine directly. A total of 30 ml of the extract at 20 mg/ml was injected into the stomach, small intestine and large intestine equally. Two hours later, collection of whole blood was performed through the guinea pig's heart. The serum samples were subjected to the inhibition test against HIV RT.

Results

Anti-HIV activity of PS
In the absence of drug, HIV-exposed cells formed numerous syncytia and died. This virus-induced CPE was inhibited in the presence of PS extract in a dose-dependent manner. This protection was total at 50 μM of PS extract. Even at this concentration, drug-induced cytotoxicity was not observed and could only be seen at over 100 μM (Figure 1). The TC_{50} value was measured as 150 μM. HIV production decreased reciprocally with the increment of
The produced Gag (p17) concentration on day 4 in culture was decreased after the addition of PS extract at 75 and 100 μg/ml and didanosine at 100 μM during HIV adsorption. However, dextran sulphate did not decrease production, even at 20 μM. At 75 μg/ml of PS extract and 20 μM of dextran sulphate, both drugs could suppress HIV production to similar levels, when added into the culture after HIV adsorption. The reduction of HIV production influenced the number of viable cells.

The production of Gag (p17) concentration decreased steeply, which indicates that the survival of HIV-exposed cells was due to the anti-HIV activity. IC_{50} and IC_{90} of PS extract, determined by the number of viable cells, was 25 and 50 μg/ml, respectively. These were generally the same as the values determined by Gag production. Anti-HIV activity was recognized for didanosine and dextran sulphate. The IC_{50} and IC_{90} values under these experimental conditions, were 5 and 20 μM for didanosine, and 0.5 and 0.8 μM for dextran sulphate, respectively. These activities are similar to those in MT-2 cells (data not shown).

Inhibition of giant cell formation
When HIV-infected U937 and uninfected MT-2 were mixed together, in the absence of drug, giant cells and balloon-shaped cytoplasm formed and could be easily visualized under light microscope 3 h after mixing. By either pretreatment of uninfected MT-2 cells or HIV-1-infected U937 cells with PS extract, morphologically altered cells were not recognized 3 h later (Figure 2).

Inhibition of proviral DNA synthesis
The PCR products of proviral DNA and the competitor (Figure 3) were visualized as two bands in the same gel lane. At the end of HIV adsorption for 1 h (Figure 3), proviral DNA could be detected at 200000 and 300000 copies/μg total DNA. The mean value of these at time 0 was subtracted from each value. Proviral DNA copies in the cells cultured in the absence of drug were more than the upper detectable limit (420000 copies) (Figure 3). Values in the presence of the drugs were 0 and 160000 for PS extract, 0 and 30000 for didanosine, and 40000 and >420000 for dextran sulphate. This experiment was performed independently twice. The representative data are shown in Figure 3.

The decreased rate of HIV production during or after HIV adsorption
The produced Gag (p17) concentration on day 4 in culture was decreased after the addition of PS extract at 75 and 100 μg/ml and didanosine at 100 μM during HIV adsorption. However, dextran sulphate did not decrease Gag production, even at 20 μM. At 75 μg/ml of PS extract and 20 μM of dextran sulphate, both drugs suppressed HIV production to similar levels when those drugs were added into the culture after HIV adsorption (Figure 4). To inhibit HIV replication by drug addition during HIV adsorption, high concentrations of drug are required even for dextran sulphate under these experimental conditions. Presumably, PS extract penetrated into the cells during the period of HIV adsorption and inhibited HIV replication.

Inhibition of HIV replication at adsorption by fractionated PS
HPLC-fractionated extract was also tested for anti-HIV activity. At the appropriate concentration (200-fold dilution), this fraction exhibited potent activity. HIV production was completely inhibited by 200-fold-diluted material without cytoxicity (Figure 5).
Figure 5. Inhibition of HIV replication at adsorption by fractionated PS extract

(a) PS purified fraction

(b) PS purified fraction, ddl

(c) Dextran sulphate

HIV production was completely inhibited by 200-fold-diluted material of fractionated PS extract. This material has anti-HIV activity without cytotoxicity (a). Pretreatment of cells at IC₅₀ gave an increased survival rate. The increased level was significantly higher for cells treated with dextran sulphate as compared to those with fractionated PS and didanosine (b). The amount of HIV from the cells pretreated with dextran sulphate at the concentration of IC₅₀ or even at IC₅₀ was significantly lower than that from cells pretreated with fractionated PS and ddl at IC₅₀ and IC₅₀ (p<0.05, fractionated PS versus dextran sulphate at IC₅₀ on day 3, by Student's t-test). Symbols: •, no drug control; ▲, fractionated PS at IC₅₀; ●, fractionated PS at IC₅₀; †, didanosine at IC₅₀; ‡, dextran sulphate at IC₅₀ and ■, dextran sulphate at IC₅₀.

Figure 6. Inhibition of HIV replication at reverse transcription non-competitively

In the presence of fractionated PS, TTP incorporation was inhibited dose-dependently. According to a Lineweaver-Burke plot, this material inhibits HIV RT activity non-competitively. Kₘ and Kᵢ values were 6.2 µM and 1.5 µg/ml, respectively.

When MT-4 cells were pretreated with dextran sulphate at the concentration corresponding to IC₅₀ (Figure 1), the pretreated cells challenged by HIV were significantly increased in comparison to untreated cells. After the challenge by HIV, cells pretreated with fractionated PS or didanosine at IC₅₀ did not grow over the levels of untreated cells. Pretreatment with these three drugs at the concentration corresponding to IC₅₀ resulted in increased survival rate. This was significantly higher for cells treated with dextran sulphate compared to those with fractionated PS and didanosine. The increased number of cells pretreated with fractionated PS was at approximately the same
Figure 7. Active component(s) in the plasma absorbed from intestine

The incorporation of TTP in the presence of plasma samples from guinea pig's intestine injected with water was evaluated by the mean of 1.2x10^6 d.p.m. with a standard deviation of 3.8x10^5 d.p.m. (n=3). The TTP incorporation level was decreased down to 9.5x10^5 d.p.m. as mean with a standard deviation of 2.0x10^5 d.p.m in the case of PS injection (n=5). The decrease in the incorporation must be influenced by the active component(s) (P = 0.05 by Student's t-test).

level as those pretreated with didanosine (Figure 5).

HIV production was initially recognized on day 3. The amount of HIV from the cells pretreated with dextran sulphate at the concentration of IC_{90} or even at IC_{50} was significantly lower than that from cells pretreated with fractionated PS or didanosine at IC_{50} and IC_{90}. Statistical analysis showed a significant difference between HIV production from cells pretreated with fractionated PS and dextran sulphate at IC_{90} on day 3 (P < 0.05, by Student's t-test, Figure 5).

Inhibition of HIV replication at reverse transcription

In the presence of HPLC-fractionated PS extract, TTP incorporation was inhibited dose-dependently. According to the Lineweaver–Burke plot, this material inhibited HIV RT activity in a non-competitive manner. K_{i} and K_{s} values were 6.2 μM and 1.5 μg/ml, respectively. The K_{i} value was determined on the assumption that 50% of the active component was successfully fractionated by HPLC (Figure 6).

Active component(s) in the plasma absorbed from the intestine

The incorporation of TTP in the presence of serum samples from guinea pigs injected with water (n=3) was evaluated and found to have a mean value of 1.2x10^6 d.p.m. with a standard deviation of 6.0x10^5 d.p.m. The TTP incorporation level was decreased down to a mean of 9.5x10^5 d.p.m. with a standard deviation of 2.0x10^5 d.p.m. in the case of PS injection (n=5). The decrease in incorporation should be influenced by the active component(s) of PS (P = 0.05 by Student's t-test) (Figure 7).

Discussion

Medicinal plants are already being investigated and appear to be a rich source of potentially useful materials in the fight against HIV. In the past 10 years, the National Cancer Institute in the USA has tested 34528 plant extracts for anti-HIV activity (Senior, 1996). The investigation of indigenous plants for use against HIV should be undertaken in every country. However, to date this strategy is far from reality. The key issue to circumvent this difficulty is to identify the active component(s) to be absorbed from the intestine to plasma.

We tested readily available plant extracts against HIV. Among nine extracts of Terminalia chebula, Syzygium aromaticum, Rhus javanica, Gruum japonicum, Alpinia officinarum, Paponia suffruticosa, Phylloladendron amurense, Panax grunaturn, and Prunella vulgaris, all, except that from Phylloladendron amurense, inhibited syncytium formation and resultant cell death caused by HIV infection to some extent (data not shown). The active component(s) may be broadly prevalent in these plants like the compound, eugenin, found in both G. japonicum and S. aromaticum (Kurokawa et al., 1998). Only an extract of PS showed potent activity, as reported elsewhere. PS is the spic of Prunella vulgaris L. var. ilicinna Nakai (Labiate). The herb is a perennial plant widely distributed through Japan, and has been used in traditional medicine as a diuretic. In Japan only the spic is used, but in China the stalk and leaves have been utilized, and in Europe the whole herb has been used. The isolation of urosolic acid, oleantaric acid and oleane tripterpene has been reported (Kojima & Oguca, 1986).

To date, several studies have been carried out on the extract and fractionated products of P. vulgaris regarding anti-HIV activity in vitro, at HIV adsorption and reverse transcription (Yamashita et al., 1998; Yao et al., 1992). In terms of clinical application, further studies are required, such as direct demonstration of the actual activity in the cells, kinetic analysis of RT inhibition, and intestinal absorption. The inhibition of provirus synthesis strongly suggests that the component can penetrate cells, and shows the inhibition of HIV replication at pre-integration step, which also supports inhibition at reverse transcription by this extract. The non-competitive inhibition indicates that the extract acts by binding to a site on the RT molecule other than the active site. This pattern is superficially similar to allosteric inhibition (Wolfe, 1993) and resembles the binding pattern of non-nucleoside reverse transcriptase inhibitors (De Clercq, 1998). The inhibitory activity against HIV reverse transcription enabled us to speculate on the intestinal absorption of the extract. We believe this

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is important for the feasibility of oral administration. Undoubtedly this kind of extract should be administrated orally.

We also reported that among those drugs tested, extracts of G. japonicum, S. aromaticum and T. chebula significantly suppressed murine cytomegalovirus in lungs of treated mice (Yukawa et al., 1996). The activity of the PS extract also acted in the same manner against cytomegalovirus (data not shown).

In this study, we recognized that a component(s) of PS inhibits HIV at reverse transcription, presumably with allosteric modification to this enzyme, like nevirapine (De Clercq, 1998), which is another mode of action in addition to inhibition at adsorption. Furthermore, guided by the impairment of this enzymatic activity, an active component(s) was detected in the plasma of guinea pigs, having entered via the intestinal tract in vivo, which shows the feasibility of oral administration. We believe that these findings will be one of the important steps in the use of indigenous plants for the treatment of HIV infection, as well as drug development.

References


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