# Tannin inhibits HIV-1 entry by targeting gp41[1]

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**KEY WORDS** tannin; drug dose-response relationship; enzyme-linked immunosorbent assay; HIV envelope protein gp41; phenols; plant extracts; medical plants

## ABSTRACT

**AIM:** To investigate the mechanism by which tannin inhibits HIV-1 entry into target cells. **METHODS:** The inhibitory activity of tannin on HIV-1 replication and entry was detected by p24 production and HIV-1-mediated cell fusion, respectively. The inhibitory activity on the gp41 six-helix bundle formation was determined by an improved sandwich ELISA. **RESULTS:** Tannins from different sources showed potent inhibitory activity on HIV-1 replication, HIV-1-mediated cell fusion, and the gp41 six-helix bundle formation. **CONCLUSION:** Tannin inhibits HIV-1 entry into target cells by interfering with the gp41 six-helix bundle formation, thus blocking HIV-1 fusion with the target cell.

## INTRODUCTION

Extracts of several Chinese medicinal herbs have been shown to have anti-HIV-1 activity, which is correlated with the concentrations of polyphenolic compounds in the extracts[1]. Our previous study has demonstrated that the polyphenolic compounds isolated from two antiviral herbs interact with the peptides derived from the HIV-1 gp41, suggesting that the active components in these herbs may inhibit HIV-1 entry by targeting gp41[2]. Tannin (also named tannic acid) is a polyphenolic compound with a potent anti-HIV-1 activity. It has been proposed that tannin inhibited HIV-1 replication by targeting the HIV-1 reverse transcriptase[3], protease[4], and intergrase[5]. It is interesting to know whether tannin also inhibits HIV-1 entry into target cells and what is the mechanism of action.

HIV-1 gp41, a transmemberane subunit of the envelope glycoprotein, plays an important role in the early steps of virus entry into target cells. After HIV-1 binding to CD4 and a coreceptor (CXCR4 or CCR5) on the target cell, gp41 changes conformation from a native state to an intermediate state, then to a post-fusion state, in which the N- and C-terminal heptad repeat regions (NHR and CHR, respectively) interact with each other to form a six-helix bundle, which brings both the viral and target cell membranes into proximity for fusion[6,7]. Peptides derived from the NHR and CHR regions, designated N- and C-peptides, respectively, such as N36 and C34, can mimic the NHR and CHR regions to form the six-helix bundle[8,9]. C-peptides can also interact with the gp41 NHR region to interfere with the interac-

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tion between NHR and CHR regions, thus blocking HIV-1 fusion[8-11]. Similar, any compound that interacts with the gp41 NHR region may also block the six-helix bundle formation and inhibit HIV-1 entry into target cells[11]. Using a monoclonal antibody (NC-1) specific for the gp41 six-helix bundle[12], we previously developed a sandwich enzyme-linked immunosorbent assay (ELISA) to screen for HIV-1 entry inhibitors targeting gp41[13] and identified the first non-peptidic HIV-1 fusion inhibitor (ADS-J1)[14]. The present study was simed to investigate whether tannin inhibited HIV-1 entry into target cells by interfering with the gp41 six-helix bundle formation.

## MATERIALS AND METHODS

**Reagents** MT-2 cells, HIV-$1_{IIIB}$ chronically-infected H9 (H9/HIV-$1_{IIIB}$) cells, and HIV-$1_{IIIB}$ were obtained from the AIDS Research and Reference Reagent Program, NIH, USA. The rabbit polyclonal antibody (PAb) and mouse monoclonal antibody (MAb) specific for the gp41 six-helix bundle were prepared and characterized as previously described[12,13]. Rabbit and mouse IgG were purified using protein-A kits (Pierce, Rockford, IL). Peptides N36 and C34 were synthesized by a standard solid-phase FMOC method in the MicroChemistry Laboratory of the New York Blood Center. The peptides were purified to homogeneity by high-performance liquid chromatography (HPLC). The identity of the purified peptides was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems). Tannins were purchased from different sources: Tannin 1 (Sigma, T-8406, product of Japan); Tannin 2 (Sigma, T-0125, product of China); Tannin 3 (Fluka, 48812, product of Switzerland); Tannin 4 (Fluka, 48811, product of the USA); Tannin 5 (Riedelde Haen, 16201, product of German). The purity of tannin 1 to 5 is 96 %, 80 %, 92 %, 92 %, and 87 %, respectively. They have a similar structure, as shown in Fig 1.

**HIV replication** The inhibitory activity of tannin on HIV-$1_{IIIB}$ replication was determined as previously described[15]. Briefly, $1\times10^4$ MT-2 cells were infected with HIV-$1_{IIIB}$ (100 $TCID_{50}$) in 200 μL RPMI-1640 medium containing 10 % FBS in the absence or presence of compounds at graded concentrations at 37 ºC for overnight. Then the culture supernatants were removed and fresh media were added. On the fourth day post-infection, 100 μL of culture supernatants were collected from each well, mixed with equal volumes of 5 % Triton X-100 and assayed for p24 antigen. The percent-

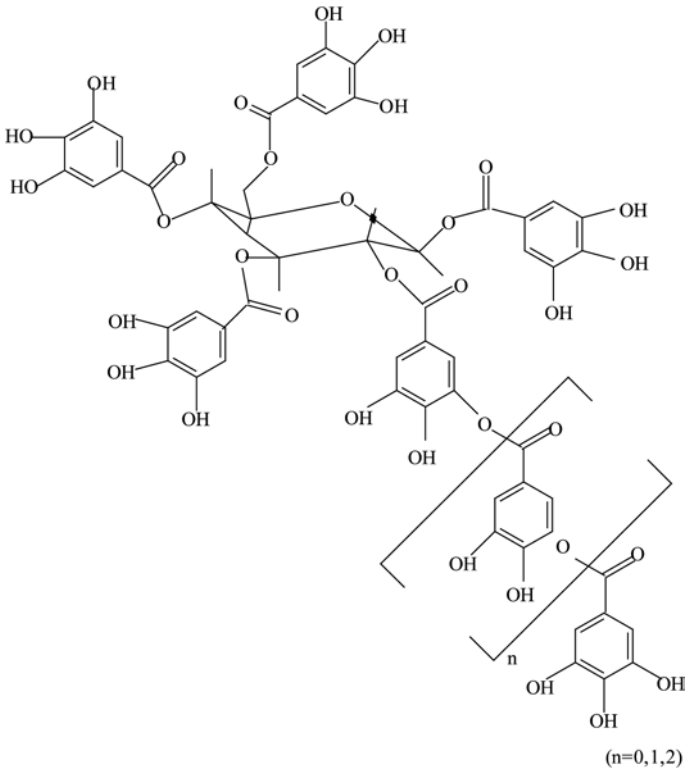


**Fig 1. The chemical structure of tannin.**

age of inhibition of p24 production and the $IC_{50}$ values were calculated as described previously[10].

**HIV-1 mediated cell fusion** A dye transfer assay was used for detection of HIV-1 mediated cell fusion as previously described[10,16]. H9/HIV-$1_{IIIB}$ cells were labeled with a fluorescent reagent, Calcein-AM (Molecular Probes, Inc, Eugene, OR) and then incubated with MT-2 cells (ratio=1:5) in 96-well plates at 37 ºC for 2 h in the absence or presence of tannin at graded concentrations. The fused and unfused Caceinlabeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc. The percentage of inhibition of cell fusion and the $IC_{50}$ values were calculated as previously described[10].

**Sandwich ELISA** A sandwich ELISA[13] was modified as described previously[17] and used for determining the inhibitory activity of tannin on the gp41 six-helix bundle formation. Briefly, peptide N36 (2 μmol/L) was preincubated with compounds at graded concentrations at 37 ºC for 30 min, followed by addition of C34 (2 μmol/L). For testing the effect of tannin on the pre-formed six-helix bundle formation, peptide N36 was preincubated with C34 at 37 ºC for 30 min before addition of tannin. After incubation at 37 ºC for 30 min, the mixture was added to wells of 96-well polystyrene plates which were precoated with NC-1 IgG (2 mg/L). Then, polyclonal antibody (IgG 5 mg/L) purified from rabbit antisera directed against the gp41 six-helix bundle[13], horseradish peroxidase-conjugated goat-anti-rabbit IgG (Boster Biotechnology Inc, Wuhan, China), and the substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) were added sequentially. Absorbance at 450 nm ($A_{450}$) was determined spectrophotometrically by an ELISA reader. The percentage of inhibition of the six-helix bundle formation and $IC_{50}$ values were calculated as previously described[13].

All the experiments were repeated at least twice. Similar results were obtained. One set of the representative data was shown here.

## RESULTS

**Tannin inhibited HIV-1 replication** Since tannin is a non-uniform polyphenolic compound, we collected tannins from different sources and compared their *in vitro* cytotoxicity and inhibitory activity on HIV-1 gag protein p24 production. As determined by the trypan blue exclusion assay, none of these tannins had detectable cytotoxicity at the final concentration of 2.5 g/L. All these tannins significantly inhibited p24 production (Tab 1, Fig 2), confirming that tannin indeed inhibits HIV-1 replication, as indicated by reduction of p24 antigen production. Their $IC_{50}$ values, ranging from 5.76 to 20.65 mg/L, are unrelated to their purity and structure. The discrepancy will be further investigated.

**Tab 1. Inhibitory activity of tannins from different sources on HIV-1 infection and the gp41 six-helix bundle formation.**

| Compound | p24 production | | Cell fusion | | Six-helix bundle formation | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ | $IC_{90}$ | $IC_{50}$ | $IC_{90}$ | $IC_{50}$ | $IC_{90}$ |
| Tannin-1 | 20.65 | 37.26 | 10.24 | 20.83 | 0.78 | 1.62 |
| Tannin-2 | 13.47 | 26.39 | 13.10 | 23.41 | 0.53 | 1.62 |
| Tannin-3 | 8.29 | 19.77 | 8.82 | 13.97 | 1.14 | 2.09 |
| Tannin-4 | 9.48 | 17.86 | 7.10 | 16.78 | 0.69 | 2.12 |
| Tannin-5 | 5.76 | 10.06 | 5.41 | 15.70 | 0.72 | 2.81 |

[1] $IC_{50}$ and $IC_{90}$: mg/L for 50 % and 90 % inhibition, respectively.

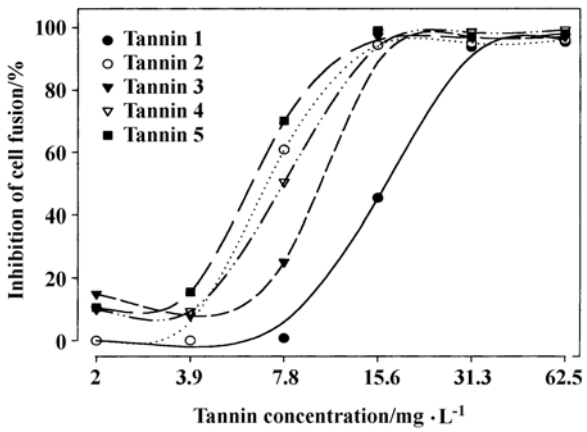


**Fig 2. Tannins from different sources inhibited HIV-1 replication.**

**Tannin inhibited HIV-1-mediated cell-fusion** Fusion of HIV-1-infected cells or HIV-1 virions with target cells is the crucial step of HIV-1 entry into target cells. Therefore, HIV-1-mediated cell fusion assays have been used for identification of HIV-1 entry inhibitors[10,16]. In the present study, tannins were tested for their inhibitory activity on HIV-1-mediated cell fusion. The results showed that all tannins from different sources inhibited fusion of HIV-$1_{IIIB}$-infected H9 cells with uninfected MT-2 cells (Fig 3) with $IC_{50}$ values varying

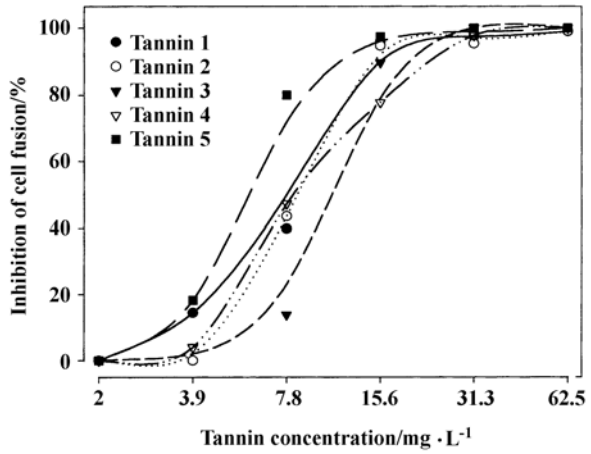


**Fig 3. Tannins blocked HIV-1 mediated cell fusion.**

from 5.41 to 10.24 mg/L (Tab 1), indicating that tannin is a potent HIV-1 entry inhibitor.

**Tannin interfered with the gp41 six-helix bundle formation** We previously developed a sandwich ELISA for identification of HIV-1 entry inhibitors targeting gp41 using synthetic peptides N36 and C34 that mimic the gp41 NHR and CHR regions and a conformation-specific MAb NC-1 which recognize the gp41 six-helix bundle[12,13]. Later, we modified this method in order to enhance its sensitivity[17]. As shown in Fig 4, MAb NC-1 did not recognize individual peptides N36 and C34, but significantly bound to the complex formed by mixing N36 and C34 at equimolar concentration. The complex formation was blocked by a small molecular HIV-1 fusion inhibitor, ADS-J1[11,14,18]. This result confirms the modified sandwich ELISA is sensitive and specific for detection of the inhibitory activity of anti-HIV-1 agents that block the gp41 six-helix bundle formation.

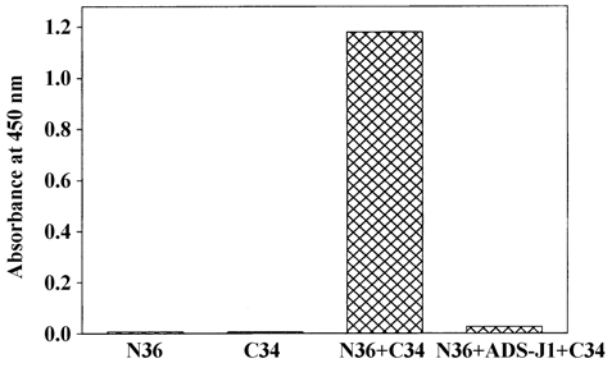


**Fig 4. Determination f the gp41 six-helix bundle formation by a modified sandwich ELISA.**

The inhibitory activity of tannins on the six-bundle formation was determined using the modified sandwich ELISA. As shown in Fig 5, all the tannins from different sources effectively inhibited the six-helix formation between the peptides N36 and C34 with $IC_{50}$ values about or less 1 mg/L (Tab 1), suggesting that tannins block the interaction between the gp41 NHR and CHR regions to form the fusion-active six-helix bundle, thus inhibiting HIV-1 fusion and entry.

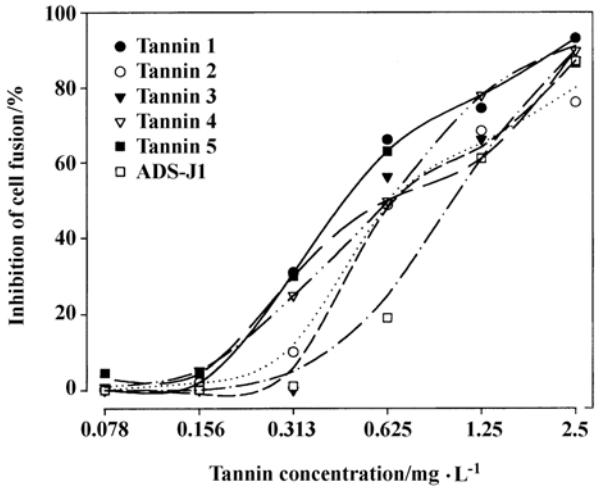


**Fig 5. Tannins inhibited the HIV-1 gp41 six-helix bundle formation in a dose-dependent manner.**

Since the six-helix bundle formation was quantitated based on the amount of the MAb NC-1 bound to the complex captured on the plate, one may argue that the reduction of absorbance at 450 nm may not be due to the inhibition of six-helix bundle formation, but rather the blockage of NC-1 binding to the six-helix bundle by tannin. To exclude this possibility, tannins were added to the pre-formed six-helix bundle before addition of the MAb NC-1. As shown in Fig 6, tannins did not interfere with NC-1 binding to the pre-formed six-helix bundle, confirming that tannin specifically interacts with a component of gp41 for inhibiting the six-helix bundle formation. Furthermore, this result also suggests that tannin cannot disrupt the pre-formed six-helix bundle, which is an extremely stable post-fusion gp41 core structure[8].

## DISCUSSION

It has been reported that tannin is a potent inhibitor of HIV-1 replication by targeting the viral proteins

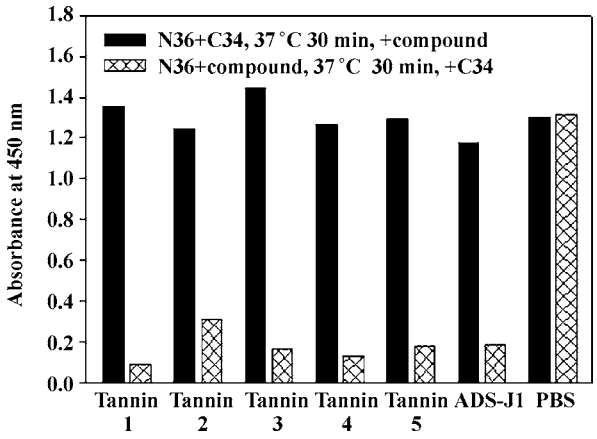


**Fig 6. Effect of tannin on the gp41 six-helix bundle formation before and after *N*- and *C*-peptide interaction.**

that mediate the late steps of HIV replication, *eg*, HIV-1 reverse transcriptase[3], protease[4], and intergrase[5]. The present study has demonstrated that tannin inhibits not only HIV-1 replication, but also HIV-1 entry into target cells as indicated by blocking HIV-1-mediated cell fusion. To study the mechanism of action, we have investigated whether tannin interferes with the formation of the gp41 six-helix bundle modeled by the N- and C-peptides derived from the NHR and CHR regions since the association of NHR and CHR to form gp41 core structure is the critical step of HIV-1 fusion. Here we showed at the first time that tannins from different sources indeed effectively inhibited the gp41 six-helix bundle formation at the concentration less than 1 mg/L. These results suggest that tannin has multiple mechanisms of action against HIV-1, *ie*, blocking HIV-1 entry by interfering with the fusion-active gp41 core formation and inhibiting HIV-1 replication by targeting reverse transcriptase[3], protease[4], and intergrase[5]. At high concentrations, tannin is able to precipitate proteins or peptides. However, we have not noticed any precipitation of the N- and C-peptides when the final concentration of tannin in the mixture reaches to 1 g/L, about 1000-folds higher than its $IC_{50}$ for inhibiting the gp41 six-helix bundle formation, indicating that the activity of tannin in this assay is not due to its non-specific interaction with peptides.

Tannin is a complex and non-uniform compound with a molecule weight more than 1700 daltons. Thus, it is unlikely to be developed as an orally applicable therapeutic for treatment of HIV-1 infection. However, it can be developed as a microbicide for topical application to prevent sexual transmission of HIV-1 because tannin is a potent anti-HIV-1 agent with multiple mechanisms of action, thus having less chance to induce drug-resistant HIV-1 mutants. Tannin is present in a variety of foods and drinks, it should have no toxic effect on human. The results obtained from the *in vitro* assay confirm that tannin has low cytotoxicity. Furthermore, tannin is abundant in herbs, vegetables, fruits, and bark of plants (especially the bark of the oak species). It is expected to be inexpensive for production. All in all, tannin has great potential to be developed as topically applicable microbicides for protecting high-risk populations from HIV-1 infection through sexual transmission.

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