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Ginkgolic acid inhibits HIV protease activity and HIV infection *in vitro*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Several HIV protease mutations, which are resistant to clinical HIV protease inhibitors (PIs), have been identified. There is a great need for second-generation PIs with different chemical structures and/or with an alternative mode of inhibition. Ginkgolic acid is a natural herbal substance and a major component of the lipid fraction in the nutshells of the *Ginkgo biloba* tree. The objective of this study was to determine whether ginkgolic acid could inhibit HIV protease activity in a cell free system and HIV infection in human cells.

Material/Methods: Purified ginkgolic acid and recombinant HIV-1 HXB2 KIIA protease were used for the HIV protease activity assay. Human peripheral blood mononuclear cells (PBMCs) were used for HIV infection (HIV-1$_{SF162}$ virus), determined by a p24gag ELISA. Cytotoxicity was also determined.

Results: Ginkgolic acid (31.2 μg/ml) inhibited HIV protease activity by 60%, compared with the negative control, and the effect was concentration-dependent. In addition, ginkgolic acid treatment (50 and 100 μg/ml) effectively inhibited the HIV infection at day 7 in a concentration-dependent manner. Ginkgolic acid at a concentration of up to 150 μg/ml demonstrated very limited cytotoxicity.

Conclusions: Ginkgolic acid effectively inhibits HIV protease activity in a cell free system and HIV infection in PBMCs without significant cytotoxicity. Ginkgolic acid may inhibit HIV protease through different mechanisms than current FDA-approved HIV PI drugs. These properties of ginkgolic acid make it a promising therapy for HIV infection, especially as the clinical problem of viral resistance to HIV PIs continues to grow.

key words: ginkgolic acid • HIV protease inhibitor • cytotoxicity • HIV infection

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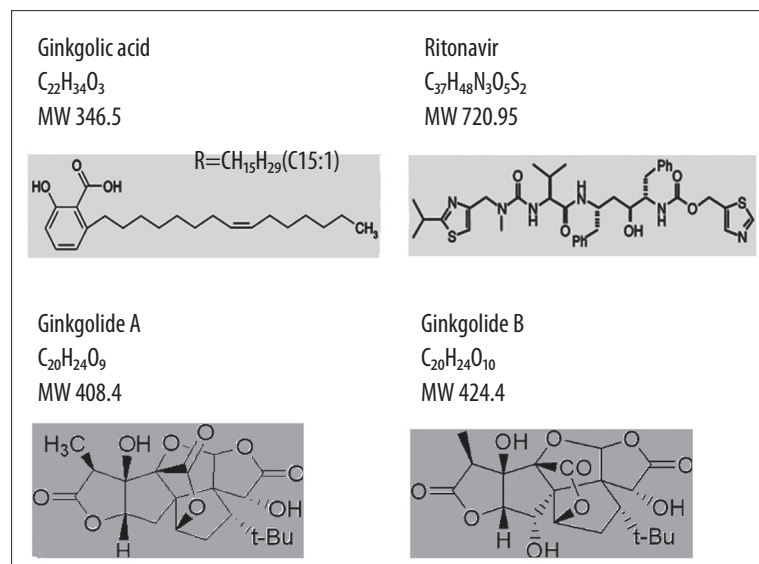
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BACKGROUND

Human immunodeficiency virus-1 (HIV-1) infection represents an ongoing global health crisis; over 1.3 million Americans and approximately 33 million people worldwide are infected [1]. Approximately 2.5 million new cases are diagnosed each year, and half of these infections occur in individuals under the age of 25 years [1]. Fortunately, since the advent of highly active antiretroviral therapy (HAART) nearly two decades ago, the morbidity and mortality associated with HIV-1 infection have become markedly reduced. However, there are important challenges now associated with HAART therapy including cardiovascular disease, cancer and rapid development of drug-resistant HIV viruses [2,3]. Potential targets for anti-HIV drugs are developed based on the HIV viral life cycle. Various drugs can inhibit virus adsorption, viral fusion, viral uncoating, or specific viral replicative enzymes such as reverse transcriptase (RT), integrase (IN) and HIV protease (PR) [4,5]. Most anti-HIV drugs are HIV reverse transcriptase inhibitors (RTIs) including the nucleotide RTIs (NRTIs) and non-nucleotide RTIs (NNRTIs). At present, there are several FDA approved HIV protease inhibitors (PIs) including saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), lopinavir (LPV), atazanavir (ATZ), tipranavir (TPV), amprevavir (APV), and fosamprevnir (FPV) [5–7]. Clinical application of RTIs and PIs in various combinations has dramatically reduced the morbidity and mortality of AIDS and has significantly improved life expectancy for HIV positive patients. Meanwhile, widespread use of PIs has led to the emergence of drug-resistant HIV proteases [2], leading to substantial numbers of HIV-infected patients on HAART regimens that are unresponsive to currently available PIs. In these patients, mutations have been found in the coding sequence of 49 of the 99 amino acids of HIV protease. Substitutions at 18 or more positions directly correlates with loss of responsiveness to PI treatment. Since the existing PIs target the active site of HIV protease and have similar structures, most of the drug-resistant HIV proteases confer cross-resistance to multiple PIs. There is a great need for second-generation PIs with different chemical structures and/or with an alternative mode of inhibition [8,9]. In addition, reduced general toxicity may be achieved. Natural herbal substances represent a great opportunity for the development of new PIs.

Chinese herbal medicines have been used for thousands of years based largely on anecdotal observations by practitioners. However, it is quite possible that certain herbal remedies may have specific therapeutic action with respect to HIV infection [10]. For example, some natural herbal substances have been shown to be effective at inhibiting the growth of HIV *in vitro* at different stages in HIV-1 replication [10,11]. Collins et al. [8] reported that 6 out of 19 aqueous herbal extracts significantly inhibited the interaction between HIV-1 gp120 and immobilized CD4 receptors. Several extracts have also been shown to be capable of inhibiting the activity of recombinant HIV-1 protease [12,13]. Extract from Ginkgo leaves is one of the most widely used herbal supplements and has become increasingly popular in recent years. Ginkgo contains two groups of active substances: flavonoid glycosides including quercetin and rutin, and terpene lactones including ginkgolides A, B, C and ginkgolic acid. The antioxidative activity of ginkgo compounds contributes to the protective effects seen in humans in multiple organ systems including visual, cardiovascular, pulmonary, and central nervous systems [14]. However, it is not known whether ginkgo compounds can affect HIV infection.

Ginkgolic acids have low cytotoxicity [15]. They are 2-hydroxy-6-alkylbenzoic acids (also known as 6-alkyl salicylic acids) with saturated or up to triple unsaturated n-C13- to n-C19- alkyl residues; the most common residues are mono-unsaturated $C_{15}H_{29}$ and $C_{17}H_{33}$. A total of 9 derivatives have been identified and exist as a mixture in ginkgo extracts. Ginkgolic acids are found in the lipid fraction of the nutshells of *Ginkgo biloba* and are also present in Ginkgo leaves. In this study, we focused on one specific compound, a simple unsaturated (R=C15:1) ginkgolic acid, which is the main component of the nutshells and leaves [16–18]. The chemical structure of ginkgolic acid is different than that of current HIV PIs and ginkgolides, though it has some similarity with aspirin (Figure 1). The objective of this study was to determine whether ginkgolic acid could inhibit HIV protease activity in the cell-free system and control HIV infection in a cell culture model.

MATERIAL AND METHODS

Reagents

Purified single compound ginkgolic acid ($C_{22}H_{34}O_3$) with 90% purity by HPLC was purchased from AXXORA, LLC (San Diego, CA). Recombinant HIV-1 HXB2 KIIA protease was generously provided by Dr. David Davis from the HIV and AIDS Malignancy Branch, National Cancer Institute at the National Institutes of Health. HIV-1 HXB2 KIIA protease was produced from *E. Coli* and had >95% purity by HPLC analysis. The specific activity was 6.3 µM/minute/mg when assayed for 5 minutes against this substrate. The molecular weight was 10,746 by mass spectrometry. One µg/ml of HIV protease was the optimal concentration for this study. Laboratory adapted HIV-1$_{SF162}$, originally isolated from the cerebrospinal fluid of patient with AIDS, was obtained from the NIH AIDS Research and Reference Reagent Program. Human peripheral blood mononuclear cells (PBMCs) of seronegative donors obtained from a local blood bank. The EnzoLyte™ 520 HIV-1 protease assay kit was from AnaSpec Co., (Fremont, CA). All other reagents were from VWR.

Determination of the kinetics of the inhibition of HIV protease activity by ginkgolic acid

The EnzoLyte™ 520 HIV-1 protease assay kit (AnaSpec Co.) was used to measure HIV protease enzyme activity and inhibition by ginkgolic acid. This protease assay kit provides a convenient assay for measuring HIV protease enzyme activity and screening HIV-1 PIs using a HiLyte Fluor™488/QXL™520 fluorescence resonance energy transfer (FRET) peptide. The sequence of this FRET peptide is derived from the native p17/p24 cleavage site on Pr^{gag} for HIV-1 protease. In the FRET peptide, the fluorescence of HiLyte Fluor™488 is quenched by QXL™520 until this peptide is cleaved into two separate fragments by HIV-1 protease. Upon cleavage, the fluorescence of HiLyte Fluor™488 is recovered, and can be monitored at excitation/emission (490 nm/520 nm). Recombinant HIV-1 HXB2 KIIA protease was used in this assay [19].

Figure 1. Chemical structures of ginkgolic acid (used in this study), the commonly used HIV protease inhibitor ritonavir, ginkgolide A, and ginkgolide B.

Determination of the effect of ginkgolic acid on the inhibition of HIV infection in cell culture

Ficoll/Hypaque-isolated human PBMCs were stimulated for 3 days in RPMI/FCS containing phytohemagglutinin (5 μg/ml). PBMCs were suspended at 10^7 cells/ml in RPMI medium only or RPMI with varying concentrations of ginkgolic acid; then, they were mixed with 10^5 $TCID_{50}$ of HIV-1_{SF162} cell free virus at the multiplicity of infection (m.o.i.) of 0.005 TCID50 per cell. After a 30 min adsorption period, sequential 2-fold dilutions of the solutions were added to cultures of PBMCs (2×10^5 PBMCs with 0.5 m.o.i of HIV-1_{SF162} virus). After that, the cells were incubated with HIV-1_{SF162} virus in a 5% CO_2 humidified incubator at 37°C for 7 days. The supernatants were harvested and analyzed for HIV-1 p24. The levels of HIV p24 antigen in the supernatant samples were assayed by a p24gag enzyme-linked immunosorbent assay according to the manufacturer's instructions (NEN Life Science Products).

Cytotoxicity of ginkgolic acid in cell culture

Jurkat cells are derived from human T-cell leukemia cells. Jurkat cells (10^6 cells/ml) were cultured in the RPMI medium with or without different concentrations of ginkgolic acid for 48 hours to test the cytotoxicity of ginkgolic acid. The cytotoxicity of ginkgolic acid was determined with the CellTiter 96® AQ_ueous Assay (Promega) which uses a tetrazolium compound (MTS) and an electron coupling reagent (PMS). MTS is chemically reduced by cells into formazan, which is soluble in the tissue culture medium. The measurement of the absorbance of the formazan can be carried out using 96 well microplates at 492 nm. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells.

Statistical analysis

All experiments were performed at least 3 times. Differences between the treated and control groups were analyzed using the Student t-test for paired data with a significance level of $P < 0.05$. The results are reported as a mean with standard error.

RESULTS

The effect of ginkgolic acid on the inhibition of HIV protease activity in a cell-free system

HIV protease enzyme activity and inhibition by ginkgolic acid was assayed with the EnzoLyte™ 520 HIV-1 protease assay kit (AnaSpec Co.) in a cell free enzyme system. HIV protease inhibitor ritonavir (10.8 μg/ml or 15 μM) was used as a positive control. Ginkgolic acid showed a potent effect on the inhibition of HIV protease activity in the cell free system (Figure 2). Ginkgolic acid (31.2 μg/ml) inhibited HIV protease activity by 60%, compared with the negative control, and the effect was concentration-dependent. In addition, the effect of ginkgolic acid on the inhibition of HIV protease activity was also compared with that of other ginkgo compounds such as ginkgolide A, which did not have any effect on HIV protease activity. Furthermore, relatively small amounts of ginkgolic acid did not change the pH in the reaction system.

The effect of ginkgolic acid on the inhibition of HIV infection *in vitro*

HIV-1_{SF162} cell free virus combined with varying concentrations of ginkgolic acid in RPMI medium were added to cultures of human PBMCs with 0.5 m.o.i of HIV-1_{SF162} virus. HIV p24 antigen in the supernatant was measured by quantitative ELISA. Ginkgolic acid treatment (50 and 100 μg/ml) effectively inhibited the HIV infection at day 7 in a concentration-dependent manner (Figure 3). However, Ginkgolides A and B at 50 μg/ml did not inhibit HIV-1_{SF162} infection in HBPCs (Figure 3). Ritonavir was used as a positive control in the experiment.

The cytotoxicity of ginkgolic acid on Jurkat cells *in vitro*

The cytotoxicity of ginkgolic acid on Jurkat cells was determined *in vitro* with the CellTiter 96® AQ_ueous Assay (Promega)
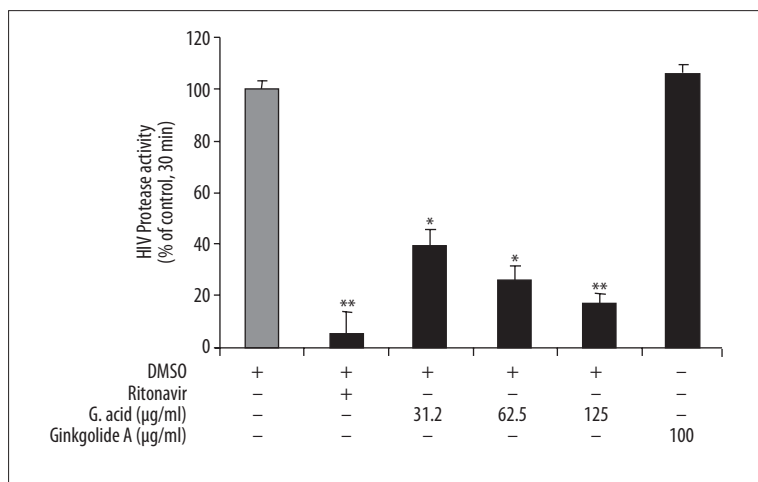
Figure 2. Ginkgolic acid inhibits HIV protease activity in a concentration-dependent manner. Recombinant HIV-1 HXB2 KIIA protease and the EnzoLyte™ 520 HIV-1 protease assay kit (AnaSpec Co.) were used in this study. HIV protease inhibitor ritonavir (10.8 µg/ml or 15 µM) was used as a positive control. Ginkgolic acid (32.2, 62.6 and 125 µg/ml) and ginkgolide A (100 µg/ml) were used. ** $P<0.01$ and * $P<0.05$ as compared with the negative control (DMSO).
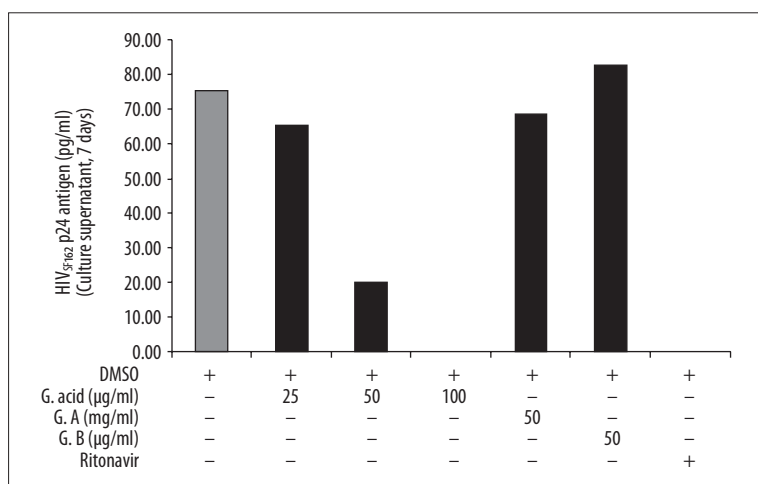

Figure 3. Ginkgolic acid, but not ginkgolide A and B, inhibits HIV infection in human PBMC cells. Human PBMCs were stimulated for 3 days in RPMI/FCS containing phytohemagglutinin (5 µg/ml). PBMCs (2×10^5 per well) were infected with HIV-1_{SF162} (0.5 m.o.i.) for 7 days. Ritonavir (15 µM), ginkgolic acid (25, 50 and 100 µg/ml), Ginkgolide A (50 µg/ml) or Ginkgolide B (50 µg/ml) was included in the culture. The levels of HIV p24 antigen in the supernatant samples were assayed by a p24gag enzyme-linked immunosorbent assay.
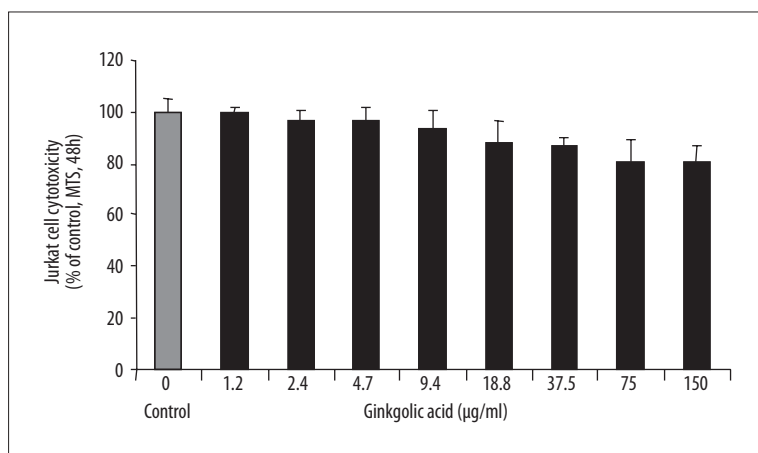
Figure 4. Ginkgolic acid at the concentrations up to 150 µg/ml did not cause any significant cytotoxicity in Jurkat cells. The Jurkat cells were cultured for 48 hours with various concentrations of ginkgolic acid. The cytotoxicity of ginkgolic acid on Jurkat cells was determined *in vitro* with the CellTiter 96® AQ$_{ueous}$ Assay.

which uses a novel tetrazolium compound (MTS) and an electron coupling reagent (PMS). The Jurkat cells were cultured for 48 hours with various concentrations of ginkgolic acid and the number of viable cells were analyzed by MTS assay. The cytotoxicity of ginkgolic acid was minimal in Jurkat cells tested *in vitro* (Figure 4). PBMCs treated with ginkgolic acid did not show any cytotoxicity characteristics (data not known).

DISCUSSION

HIV-1 protease plays an essential role in the life cycle of HIV because it cleaves the newly synthesized polyproteins to yield viral structural and functional proteins necessary for maturation. Thus, inhibitors of HIV-1 protease are very effective antiviral drugs that can significantly prolong the life of patients with AIDS [20]. The current PIs, however,

have several unwanted side effects. Ritonavir, for example, may cause asthenia, malaise, diarrhea, nausea and vomiting, abdominal pain, dizziness, insomnia, sweating, taste abnormalities, and problems related to metabolism [21]. As such, the development of new inhibitors of HIV-1 protease is an urgent task.

In a previous paper, Lee et al. [22] reported that ginkgolic acid (C15: 1) and several other compounds from *Ginkgo biloba* exhibited potent dose-dependent inhibitory activities on HIV-1 protease with an IC_{50} 24.9 µM, which was assayed in a cell-free system by HPLC with the synthetic heptapeptide [His-Lys-Ala-Arg-Val-Leu-(pNO2-Phe)-Glu-Ala-Nle-Ser-NH2] as the substrate. In this study, we assayed the HIV-1 inhibition activity of ginkgolic acid with a convenient HIV-1 protease assay kit in a cell free system; furthermore, we investigated the inhibitory action of ginkgolic acid on HIV-1 infection and its cytotoxicity in Jurkat cells. Our data demonstrate that ginkgolic acid effectively inhibits *in vitro* HIV infection in PBMC cells with limited cytotoxicity. Ginkgolic acid showed a potent effect on the inhibition of HIV protease activity in a concentration-dependent manner in the cell free system. Importantly, ginkgolic acid at a concentration up to 150 µg/ml had very limited cytotoxicity in Jurkat cells tested *in vitro*.

In this study we demonstrate that ginkgolic acid is able to inhibit HIV protease activity in a concentration-dependent manner, with an estimated IC_{50} of less than 30 µg/ml in the cell free system. This effect was specific; other ginkgo compounds such as ginkgolide A did not have any effect on HIV protease activity. Furthermore, the effect was not caused by pH change from the addition of ginkgolic acid. Therefore, the strong inhibitory effect of ginkgolic acid is possibly related directly to its unique chemical structure. Ginkgolic acid has a structure distinct from that of ritonavir and other current PIs, which all target the active site of HIV protease and can confer cross-resistance to multiple PIs.

Furthermore, ginkgolic acid treatment effectively inhibited the HIV-1$_{SF162}$ infection in human PBMCs in a concentration-dependent manner; ginkgolide A and ginkgolide B (50 µg/ml) had no effects on HIV infection. This confirmed that the HIV inhibition is related to the unique structure of the ginkgolic acid.

Although some reports indicate that ginkgolic acid may have allergenic, cytotoxic, mutagenic and carcinogenic effects [15,23], systematic and detailed investigations of these events in animal studies and human observations have not been performed. In general, all compounds may have both therapeutic effects and side effects, which must be weighed in the development of clinically useful drugs. Clinical studies using Ginkgo extract with less than 5 ppm ginkgolic acid have shown Ginkgo to be remarkably free of side effects. However, the side effect profile for Ginkgo exceeding this standard is not known. Our data showed ginkgolic acid was an effective HIV protease inhibitor in both a cell free system and cell culture model, with limited cytotoxicity even at concentration up to 150 µg/ml.

Conclusions

In conclusion, our preliminary data indicate that ginkgolic acid is a HIV protease inhibitor. This is a good start in the investigation of new PIs, especially in light of the growing problem of PI-resistant HIV infection, and further investigation is warranted. Several critical questions must be addressed, such as the kinetics of inhibition and the time course of the effect of ginkgolic acid on HIV infection in cell culture. Also, inhibition of several HIV strains including lab adapted strains and clinical isolates of HIV should be assessed, as well as cytotoxicity in other cell types. Bioavailability, pharmacokinetics and toxicity should be studied in animal models. Finally, a strategy to modify the chemical structure of ginkgolic acid to enhance its therapeutic effects and reduce toxic effects should be examined.

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