Original article

HIV type-1 entry inhibitors with a new mode of action

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Background: The development of antiviral drugs has provided crucial new means to mitigate or relieve the debilitating effects of many viral pathogens. Regular use of these drugs has led to generation of resistant strains, making the control of many viral infections very difficult, particularly in HIV-seropositive and AIDS patients. A rich source for the discovery of new HIV infection inhibitors has been, and continues to be, the 'mining' of the large diversity of compounds already available in nature, and specifically those from botanical extracts.
Methods: Using a newly developed direct binding assay with mass spectrometry technology (direct analysis in real-time time-of-flight mass spectrometry), we were able to show that compounds present in extracts of elderberry, cinnamon and green tea bind to and block HIV type-1 (HIV-1) infection in target cells.
Results: The compounds that blocked HIV-1 infection were flavonoids and A-type proanthocyanidins. The 50% inhibitory concentration values of these extracts ranged from 0.5 to 201 μg/ml for four different HIV-1 serotypes. Interaction matrices with the elderberry extract and enfuvirtide, a peptide HIV-1 fusion inhibitor, revealed significant super additive effects. This indicates that the compounds in elderberry that prevent HIV-1 infection are likely to bind to viral glycoproteins other than gp41 (the binding site for enfuvirtide).
Conclusions: Optimized elderberry, green tea and cinnamon extracts rich in certain flavonoid compounds were shown to block HIV-1 entry and infection in GHOST cells. As such, these types of botanical extracts could provide a starting point for the development of possible safe and reliable cotherapies for HIV-1-positive individuals, as well as for the identification of new small molecules as leading drug candidates for HIV-1 therapeutics and microbicides.

Introduction

In recent years, the development of antiviral drugs has provided crucial new means to mitigate or relieve the debilitating effects of many viral pathogens [1]. The discovery of zidovudine as an effective disrupter of the HIV type-1 (HIV-1) viral cycle has improved the quality and extended the lives of many HIV-1-positive individuals [1]. Unfortunately, the regular use of zidovudine, as well as other HIV-1 reverse transcriptase inhibitors, protease inhibitors and highly active antiretroviral therapy (HAART) involving multidrug therapies, has led to the selection of resistant HIV-1 strains, making the control of HIV-1 viral load in seropositive and AIDS patients difficult [2]. For this reason, the need for new HIV-1/AIDS therapies targeting new and unique steps in the processes leading to HIV-1 infection is a well recognized imperative for this global public health crisis [1]. Enfuvirtide (also termed T-20 or Fuzeon®), a small peptide designed on the basis of the C-terminal heptad repeat region of the viral envelope glycoprotein gp41, is one of the first products developed to fulfil this need. Enfuvirtide targets the interaction of the HIV-1 virus with CD4-carrying T-cells, the preferred target of HIV-1 [3,4], disrupting a process known as 'gp41 zipping' and thus effectively blocking the HIV-1–host cell fusion process [5]. However, research addressing small peptide homologues of the gp41 or gp120 glycoprotein regions have yet to yield viable drug leads.

Instead of designing and synthesizing *de novo* drug leads, an alternative approach to the discovery of new HIV-1 infection inhibitors is 'mining' the large diversity of compounds already available in nature, particularly those in botanical extracts. Historically, botanicals have yielded highly effective drugs (for example,

aspirin and Taxol®) [6,7] and are the foundation of traditional Chinese medicines and Ayurvedic medicines [8]. In particular, anti-HIV-1 activity of botanical extracts *in vitro* has been reported for a range of botanicals, including green and black teas, cloves, mountain rose, Damask rose, Japanese honey locust, *Tripterygium wilfordii*, echinacea, ginseng and chrysanthemum, as well as red and brown algae [9–12]. Carraguard®, a red algal sulfonated polysaccharide, has been evaluated for use as a topical anti-HIV-1 microbicide [13–15]. Mahmood *et al*. [11,16] showed that many flavonoids, including quercetin, kaempferol and epigallocatechin gallate (EGCG), inhibited HIV-1 infection through disruption of the CD4–gp120 interaction, whereas recent studies have shown that EGCG, a polyphenol common in green and black teas, binds to host cell CD4 receptors (the gp120 glycoprotein binding site of HIV-1) and prevents HIV-1 virion binding to and entry into host cells [17,18].

Viral entry inhibitors offer new and promising means to address HIV-1 infections from a therapeutic and from a topical microbiocide perspective because they address a new therapeutic target. In the present study, we utilized new methods developed for influenza viruses [19] to identify small molecules from botanical extracts that bind to the surface of HIV-1 virions and establish how such binding functions to block the viral capture and entry that was observed. In addition, we sought to identify the possible binding sites by conducting analyses on an interaction matrix with the known HIV-1 entry inhibitor, enfuvirtide. We show, for the first time, that optimized and dose-reliable extracts of elderberry, Japanese green tea and cinnamon contain bioactives that inhibit HIV-1 entry *in vitro*. This mode of action was validated using a direct binding assay that utilizes direct analysis in real-time (DART) time-of-flight (TOF) mass spectrometry (MS) to determine the chemical species present in complex botanical extracts that bind to the surface of HIV-1 virions. Furthermore, we show that when the identified bioactives are bound to the HIV-1 particles, the viruses are unable to infect host cells. Among the hundreds of compounds in the elderberry, cinnamon and green tea extracts utilized here, only two compounds were found to bind to the HIV-1 virus particles.

Methods

Botanical extracts

The elderberry extract used in this study was chosen because of its effective inhibition of influenza virus (H1N1) entry and infection *in vitro* [19]. In addition, elderberry is rich in polyphenolics [20]. Recent studies [20,21] have shown the antiviral effectiveness of small molecule bis-phenolics and the anti-HIV-1 activity of proanthocyanindins (PACs). The extraction strategy aimed to ensure that generated extracts were rich in polyphenolics in general, and flavonoids in particular; therefore, extracts were tailored to meet this requirement. Briefly, elderberry fruits (*Sambucus nigra* L.) were extracted with 80% (v/v) ethanol in two stages (2 h in each stage) at 70°C. The extracted slurry was filtered, centrifuged and the supernatant was collected and vacuum-distilled. This material was loaded onto an ADS5 polymer adsorbent column (Nankai University, Tianjin, China), washed with distilled water and then eluted with 80% (v/v) ethanol. The eluate was taken to dryness.

The cinnamon extract was chosen because it is well known to be rich in polyphenolics and detailed chemical analyses revealed many similarities to the chemical composition of elderberry (BR, Jr and RSA, unpublished data). Again, as with elderberry, extraction methods addressed the requirement for enrichment in flavonoids and polyphenolics. Cinnamon (*Cinnamon cassia*) bark was extracted using 80% (v/v) ethanol at 40°C. The extraction was performed in two stages for 2 h in each stage and the supernatants were collected and the ethanol removed. This material was loaded onto an Amberlite® XAD 7HP column (Acros Organics, Morris Plains, NJ, USA), washed with distilled water and eluted with 80% (v/v) ethanol. The eluate was taken to dryness and then loaded onto a Sephadex® LH-20 column (Amersham Bioscience AB, Uppsala, Sweden), and eluted with 95% (v/v) ethanol. The LH-20 eluate was taken to dryness under vacuum at 50°C.

The Japanese green tea extract used in this study was selected on the basis of high EGCG content and of previous studies in which antiviral efficacy was established with green tea extracts or its major phenolic components [11,16]. The extraction methods were designed to ensure a rich flavonoid and polyphenolic diversity. Green tea (*Camellia sinensis*) leaves were extracted using 60% (v/v) ethanol at 40°C. The extraction was performed in two stages for 2 h in each stage. The supernatants were collected and the ethanol was evaporated under pressure. The resulting solution was loaded onto the Amberlite® XAD 7HP adsorbent (Acros Organics, Morris Plains, NJ, USA), washed with distilled water and with 5% (v/v) sulfuric acid to remove caffeine. Distilled water was used for a final washing step and the extract was eluted with 80% (v/v) ethanol. The resulting extract was collected after being dried under vacuum at 50°C.

For the viral inhibition assays, these extracts were prepared into stock solutions and diluted appropriately as described below.

DART™ AccuTOF™ MS and structural analyses

The botanical extracts, PAC B_2 (reference standard; Chromadex, Irvine, CA, USA) and viral-bound compounds

were analysed using a JMS-T100 DART™ AccuTOF™ Mass Spectrometer (JEOL USA, Peabody, MA, USA). The settings for the DART ionization source were needle voltage =3,500 V, temperature =300°C, electrode one =150 V, electrode two =250 V and helium gas flow =3.49–3.89 l/min. For the mass spectrometer, the settings loaded were orifice one =20 V, ring lens voltage =5 V, orifice two =5 V, the peaks voltage =1,000 V and the microchannel plate detector =2,550 V. Calibrations were performed internally with each sample using a 10% solution of PEG 600 (Ultra Chemicals, North Kingston, RI, USA) providing mass markers throughout the required mass range of 100–800 atomic mass units (AMU). The botanical extracts were introduced into the DART He plasma as powders using the closed end of a borosilicate glass melting point capillary tube. The capillary tube was held in the He plasma until the signal disappeared from the total ion chromatogram and the signal-to-noise ratio returned to baseline values. Molecular formulas were confirmed using elemental composition and isotopic ratio matching programmes provided by JEOL with the DART™ AccuTOF™ Mass Spectrometer [22].

Pseudotyped HIV-1 production

Pseudotyped HIV-1 virions of subtypes B and C were produced by cotransfecting 293T cells grown in T75 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM; pH 7.4, 10% [v/v] fetal bovine serum [FBS]; Gibco-Invitrogen, Carlsberg, CA, USA) with 6 µg of pSG3$^{\Delta env}$ (a plasmid containing an envelope-deficient copy of the genome of HIV-1 strain SG3 [23]) and 2 µg of the envelope clones 11023 (subtype B1), 11038 (subtype B2), 11312 (subtype C1) and 11313 (subtype C2) [24]. Effectene Transfection Reagent (Qiagen, Valencia, CA, USA) was used to transfect the cells. After 18 h, the culture medium with Effectene Transfection Reagent was replaced with fresh medium. Supernatants were collected 48 h post-transfection, clarified by low-speed centrifugation, aliquoted and frozen at -80°C. The titre of the viral stocks was determined by infecting GHOST cells [25] seeded on a 96-well plate for 2 h at 37°C with serial dilutions of virus. After the 2 h incubation, the medium with the virus was replaced with fresh DMEM containing 10% (v/v) FBS and the appropriate selective agent, and incubated for 48 h at 37°C. The GHOST cells possessed a green fluorescent protein (GFP) expression system that was initiated upon viral replication and reported viral infection. The plate was scanned and cells expressing GFP were counted using a Typhoon phosphoimager with ImageQuant software (Amersham Bioscience, Piscataway, NJ, USA).

Foci reduction assays

To assay extract activity against HIV-1, 5×10^4 GHOST cells were plated in each well of a 96-well tissue culture plate and incubated at 37°C for 18–24 h. Following the incubation, 300–1,000 focus-forming units of pseudotyped virus were added to each well in the presence of different botanical extract concentrations with the control wells containing only DMEM additions. After 2 h of incubation at 37°C, the virus- and extract-containing medium was removed, and 200 µl of DMEM containing 10% (v/v) FBS was added per well and incubation was continued for an additional 48 h at 37°C. After this incubation period, the plates were scanned and viral foci were counted using a Typhoon phosphoimager with ImageQuant software (Amersham Bioscience).

Direct binding assays

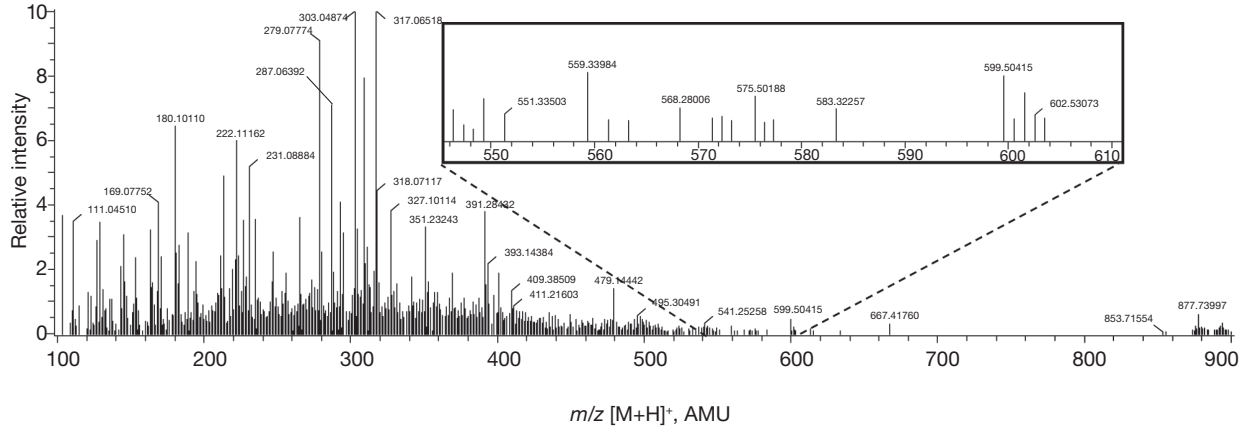
To determine compounds from the botanical extracts that bind to the envelope of HIV-1 particles, the virions were incubated in 200 µl of extract (1 mg/ml) for 1 h at room temperature following the methods of Roschek *et al.* [19]. The solutions containing the viruses were ultrafiltered using a 100 kDa molecular weight Amicon cutoff filter (Millipore, Billerica, MA, USA) followed by centrifugation at 9.3 *g* for 15 min at room temperature. The virus particles were washed with 500 µl DMEM (pH 7.4) and centrifuged under the same conditions for an additional 15 min to remove any unbound chemicals. The viral fraction with bound compounds was collected. A portion of the viral fraction was resuspended in 100 µl neat USP ethanol to inactivate the virus for DART TOF MS analysis to identify the bound compounds, whereas another portion was used in post-infection assays [19].

Interaction matrices

An interaction matrix commonly used to look at drug interactions was set up to examine drug synergies with the elderberry extract and enfuvirtide, following previously described experimental procedures and data analysis methods [11,26,27]. The experiment included 36 samples, 11 of which were controls (5 doses of enfuvirtide alone, 5 doses of the elderberry extract alone and 1 well with no chemistries, for example, extract and/or enfuvirtide as a positive control and an experimental reference for the inhibition curves) and 25 of which were drug combinations (enfuvirtide and elderberry extract). The five concentrations tested for enfuvirtide were 1:4 serial dilutions starting from a concentration of 0.1 mg/ml, whereas the five concentrations of elderberry extracts were 1:4 serial dilutions starting from a concentration of 1 mg/ml. Drug treatment and foci formation reduction assays were performed as described above.

The data were analysed following the methods of Fairbanks and Wilcox [27] to determine whether competitive, additive or super additive/synergistic

Figure 1. Chemical characterization of the elderberry extract



Positive ion [M+H]+ direct analysis in real-time time-of-flight mass spectrometry fingerprint of the elderberry extract. AMU, atomic mass units.

interactions occurred. Briefly, the potencies of the elderberry extract relative to enfuvirtide were calculated as the ratio of the 50% inhibitory concentration (IC_{50}) values of the extract over the IC_{50} values of enfuvirtide. The concentrations of elderberry extract equivalent to the enfuvirtide doses were determined by dividing the concentrations over the potencies and summed to the actual enfuvirtide doses to give the additive doses. These doses were used to estimate the expected inhibitory values using the elderberry-extract-only inhibition curves and the expected inhibitory values using the enfuvirtide-only inhibition curve, assuming in both cases that the elderberry extract mode of action was the same as enfuvirtide.

GHOST-cell-adsorbing antiviral activity

GHOST cells were incubated in the presence of the green tea extracts at varying concentrations for 2 h at 37°C. The supernatant, containing compounds from green tea that did not adsorb to the GHOST cells, was removed and used in a foci reduction assay following the methods described earlier. Fresh HIV-1 virions and growth media were added to the original GHOST cells, containing adsorbed compounds from the green tea extract, and incubated for an additional 48 h following the methods described earlier for the foci reduction assays. The plates were scanned and viral foci counted using a Typhoon phosphoimager with ImageQuant software (Amersham Bioscience).

Cellular toxicity

Possible extract toxicity to the target cells was evaluated using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay measuring mitochondrial activity of the GHOST cells after being exposed to the extracts [28]. Briefly, cells were grown overnight in a tissue culture incubator in 96-well tissue culture plates. Extract solutions were added to the wells containing GHOST cells and incubated at 37°C for 12 h. This exposure was 6× longer than the exposure period of GHOST cells to the extract. After this incubation period, the GHOST cells were exposed to MTT for 4 h. Media was aspirated and replaced with crystal solubilization solution to distribute the formazan evenly in the well. MTT is metabolized to formazan by mitochondria of living cells. The presence of formazan is thus indicative of mitochondrial activity. Formazan was quantified in a plate reader by reading the absorbance of each well at 570 nm.

Results

Elderberry extract possess significant chemical diversity

The characterization of the elderberry extract by DART TOF MS analysis showed a large chemical diversity (>1,000 chemical species) in the mass range of *m/z* $[M+H]^+$=100–1,000 AMU (Figure 1). The mass spectrum shows the relative abundance of each chemical species detected (Figure 1, y-axis). The elderberry extract was enriched in a variety of known flavonoids including quercetin at *m/z* $[M+H]^+$=303.049 AMU and anthocyanins, such as cyanidin (*m/z* $[M+H]^+$=287.064 AMU) and petunidin (*m/z* $[M+H]^+$=317.065 AMU).

Elderberry extract blocks HIV-1 infection *in vitro*

To evaluate the anti-HIV-1 activity of the elderberry extract, an HIV-1 foci reduction assay using four

different clones of pseudotyped enveloped HIV-1 virions and GHOST cell targets (enriched in CD4 and CCR5 receptors) was employed [29]. Dose-dependent inhibition of HIV-1 infection was observed with the extracts (Figure 2). The IC_{50} of the elderberry extract for HIV-1 infection ranged from 36 to 201 μg/ml, whereas 100% inhibitory concentration (IC_{100}) values ranged from 662 to 951 μg/ml for the different HIV-1 clones (Table 1).

Toxicity evaluation of elderberry, green tea and cinnamon extracts

Mitochondrial activity as a measure of cell toxicity of the GHOST cells was determined in an MTT assay. GHOST cells were exposed to serial dilutions of the elderberry, cinnamon and green tea extracts up to concentrations of 2,000 μg/ml for 24 h. No cellular toxicity was observed for any extract under the experimental conditions reported here where the exposure periods were 6× as long as the experimental exposure periods (unpublished data).

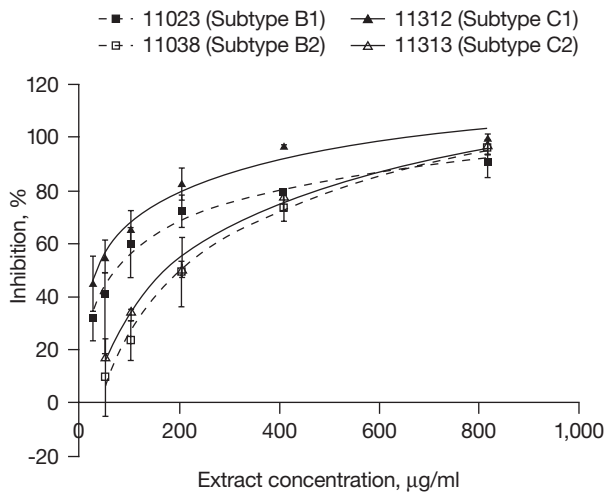
Compounds in elderberry bind to HIV-1 virions

A direct binding assay coupled with the use of DART TOF MS was utilized in order to detect and identify compounds that bind to HIV-1 virions [19], and to test the hypothesis that flavonoids present in the extract bind to HIV-1 virions and, in doing so, block infection *in vitro*. The direct binding assay allowed for DART TOF MS fingerprints to be generated and revealed the chemistries bound to the surfaces of biological or man-made materials with high sensitivity and reliability. DART TOF MS fingerprints were generated on HIV-1 particles incubated in the elderberry extract, which were washed free of unbound chemistries, at the determined IC_{50} and IC_{100} concentrations (Table 1 and Figure 3).

Molecular ions at *m/z* $[M+H]^+$=313.278, 331.289, 341.310, 359.324, 369.368, 579.524 and 607.552 AMU were detected (Figue 3A, arrows) by DART TOF MS on the surface of the HIV-1 particles. The initial wash fraction contained nearly all of the chemical species present in the elderberry extract and none of the bound compounds (Figure 3B). JEOL DART TOF MS software programmes [30] and chemical fragmentation analyses were used to determine candidate molecular formulae and structures of the HIV-1-bound compounds.

The DART TOF MS peak detected at *m/z* $[M+H]^+$=359.324 AMU (fragments *m/z* $[M+H]^+$=313.278, *m/z* $[M+H]^+$=331.289 and *m/z* $[M+H]^+$=341.310 AMU)

Figure 2. Elderberry extract infection inhibition



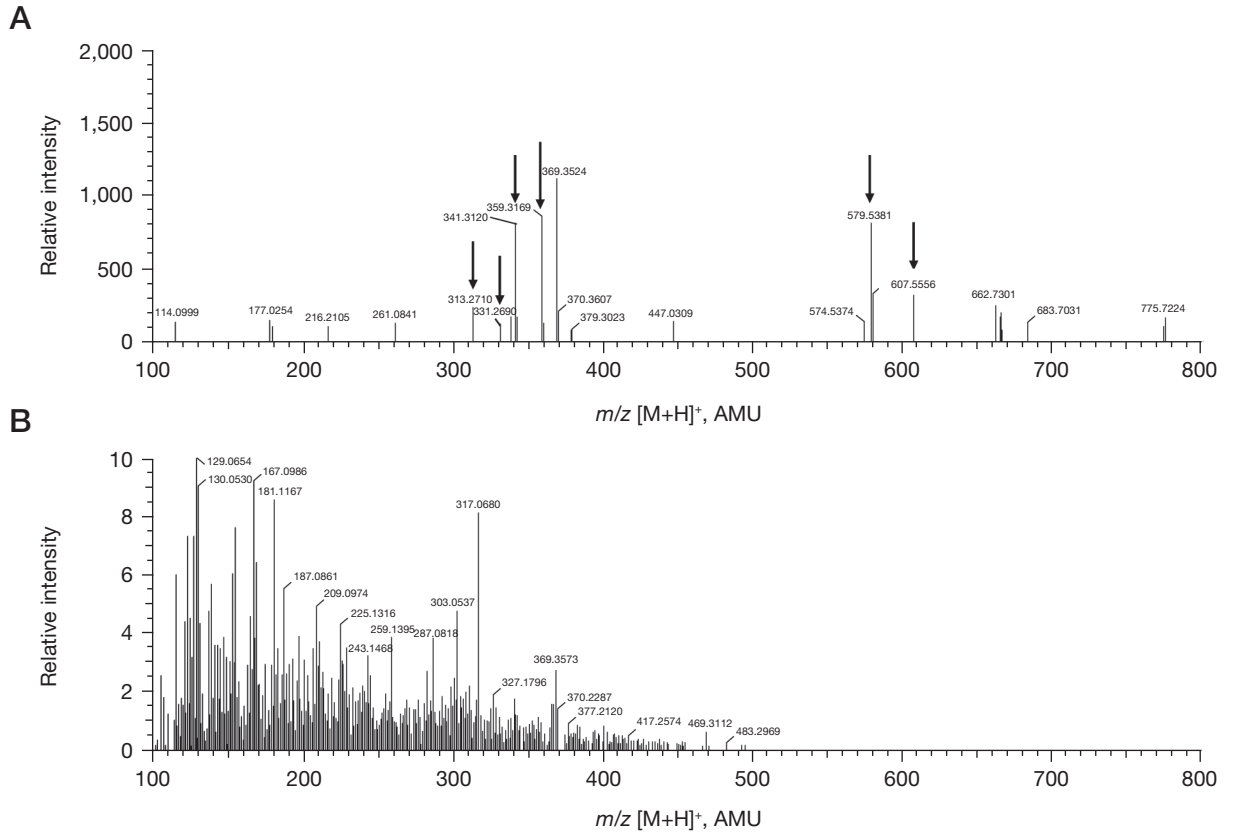
HIV type-1 (HIV-1) inhibition curves from foci reduction assays using pseudotyped HIV-1 subtypes B and C. Clones were produced by cotransfecting 293T cells with $pSG3^{\Delta env}$, a plasmid containing an envelope-deficient copy of the genome of HIV-1 strain SG3, and envelope clones 11023 (subtype B1), 11038 (subtype B2), 11312 (subtype C1) and 11313 (subtype C2), which were then used to infect GHOST cells (containing a green fluorescence protein [GFP] expression system) in the presence of the elderberry extract. The GFP-expressing GHOST cells were counted using a Typhoon phosphoimager with ImageQuant software (Amersham Bioscience, Piscataway, NJ, USA).

Table 1. The IC_{50} and IC_{100} values of elderberry, cinnamon and Japanese green tea extracts evaluated in foci reduction assays using pseudotyped HIV-1 subtypes B and C

	Elderberry, μg/ml		Cinnamon, μg/ml		Japanese green tea, μg/ml		Fuzeon®, μg/ml	Elderberry, μg/ml	Synergy elderberry versus Fuzeon®, μg/ml	
HIV-1 pseudotype	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{50}	Theoretical	Observed
11023 (Subtype B1)	70	NA	1.6	12.1	0.5	38	0.5[a]	23.1[a]	12[a]	6.9×10^{-6}[a]
11038 (Subtype B2)	201	951	1.1	25.4	5.4	108				
11312 (Subtype C1)	36	662	0.7	11.7	0.8	18	2.8[b]	89.2[b]	46[b]	5.0×10^{-3}[b]
11313 (Subtype C2)	169	936	33	106	3	62				

The HIV type-1 (HIV-1) clones were produced by cotransfecting 293T cells with $pSG3^{\Delta env}$, a plasmid containing an envelope-deficient copy of the genome of HIV-1 strain SG3, and envelope clones 11023 (subtype B1), 11038 (subtype B2), 11312 (subtype C1) and 11313 (subtype C2), which were then used to infect GHOST cells that contained a green fluorescent protein (GFP) expression system. The GFP-expressing GHOST cells were counted using a Typhoon phosphoimager with ImageQuant software (Amersham Bioscience, Piscataway, NJ, USA). [a]Value for both subtype B1 and B2. [b]Value for both subtype C1 and C2. IC_{50}, 50% inhibitory concentration; IC_{100}, 100% inhibitory concentration; NA, not achieved.

Figure 3. Elderberry extract direct binding assay



Positive ion $[M+H]^+$ direct analysis in real-time (DART) mass spectra of chemicals in the elderberry extract **(A)** bound to HIV type-1 (HIV-1) virions and **(B)** not bound to HIV-1 virions. The virions were incubated for 1 h in the extract, filtered onto a 100 kDa Amicon membrane (Millipore, Billerica, MA, USA) and washed twice with phosphate-buffered saline (pH 7.2) to remove unbound chemistries prior to DART time-of-flight mass spectrometry fingerprinting. Arrows indicate the parent ions and fragments of the identified compounds. AMU, atomic mass units.

has been determined to be 5,7,3′,4′-tetra-*O*-methyl quercetin (TMQ) [19] (Figure 3). The detected peak at *m/z* $[M+H]^+$=369.368 AMU (Figure 3) was identified as cholestadiene, a common sterol present in the virus incubation medium and our controls showed that cholestadiene did not have anti-HIV-1 activity. The proposed structure of the compound present at *m/z* $[M+H]^+$=607.552 AMU (fragment *m/z* $[M+H]^+$=579.524 AMU) is an A-type PAC that likely exists in two possible isomeric forms (Figure 4). This structure is supported by fragment analysis of the *m/z* $[M+H]^+$=607.552 AMU parent ion (Figure 4). The structural isomers share a common C3→C7 O linkage, but differed in their C4→C8 (Figure 4A) and C4→C6 linkages (Figure 4B).

DART TOF MS analysis was also conducted on PAC B_2. The PAC B_2 parent ion at *m/z* $[M+H]^+$=579 AMU (confirmed by electronspray ionization MS; data not shown) was not detected by DART TOF MS; however, DART TOF MS did show the monomeric units of PAC B_2 at *m/z* $[M+H]^+$=291 AMU. It is reasonable to conclude that B-type PACs are not stable under the DART conditions used here, supporting the assignment of the *m/z* $[M+H]^+$=607.552 AMU chemical species found bound to the HIV-1 virions as an A-type PAC. PAC B_2 yielded IC_{50} values between 200 and 350 μg/ml (2–3-fold less effective than the elderberry extract), whereas IC_{100} values were never achieved.

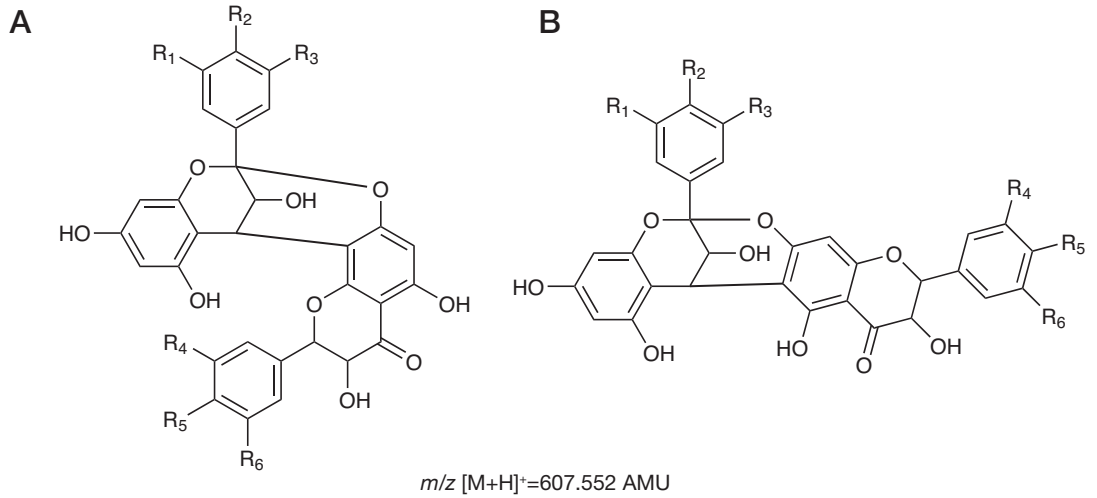
Binding to HIV-1 virions block infection

To further validate the anti-HIV-1 activity of the identified compounds, the HIV-1 particles were incubated in the elderberry extract at the determined IC_{50} and IC_{100} concentrations (Table 1) and then subjected to foci reduction assays after the unbound compounds were washed off the virions using the direct binding assay. Dose-dependent inhibition of HIV-1 entry was obtained with IC_{50} and IC_{100} values similar to those obtained from the extract alone (60% and 80% inhibition, respectively; Figure 5).

Elderberry extracts are synergistic with enfuvirtide

The IC_{50} values for enfuvirtide inhibition of HIV-1 infection alone were 0.5 and 2.8 μg/ml for the B and

Figure 4. Structures of an A-type proanthocyanidin common to elderberry and cinnamon that binds to HIV-1 virions



Substitution	Proanthocyanidin 1	Proanthocyanidin 2	Proanthocyanidin 3	Proanthocyanidin 4	Proanthocyanidin 5	Proanthocyanidin 6
$-R_1$	-OH	-OH	-OH	-OH	-OH	-H
$-R_2$	-OH	-OH	-OH	-OH	-H	-OH
$-R_3$	-OH	-OH	-OH	-H	-OH	-OH
$-R_4$	-OH	-OH	-H	-OH	-OH	-OH
$-R_5$	-OH	-H	-OH	-OH	-OH	-OH
$-R_6$	-H	-OH	-OH	-OH	-OH	-OH

m/z $[M+H]^+$=607.552 AMU —(-CO)→ *m/z* $[M+H]^+$=579.530 AMU —(-CO)→ *m/z* $[M+H]^+$=551.551 AMU

Two possible structural isomers of the A-type proanthocyanidin at *m/z* $[M+H]^+$=607.552 atomic mass units (AMU) are shown, **(A)** one with a C4→C8 linkage and **(B)** one with a C4→C6 linkage. The table shows the possible R-groups for these structural isomers and the direct analysis in real-time time-of-flight mass spectrometry generated fragments are included; *m/z* $[M+H]^+$=579.530 AMU ($[M+H]^+$-CO) and *m/z* $[M+H]^+$=551.511 AMU ($[M+H]^+$-2×CO). HIV-1, HIV type-1.

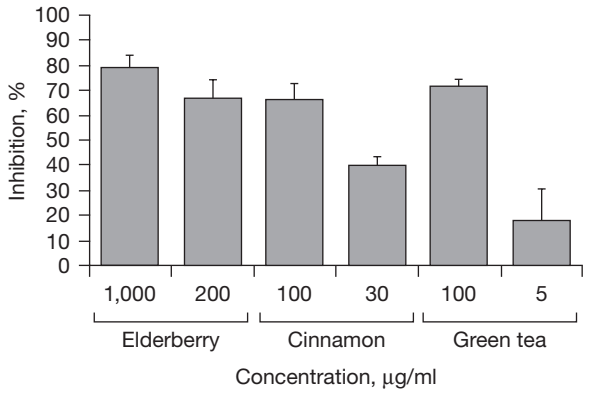
C subtype HIV-1 clones, respectively (Table 1). When enfuvirtide was combined with the elderberry extract, a high level of synergy was obtained. The IC_{50} values decreased from 0.5 and 2.8 μg/ml to 6.9×10^{-6} and 5.0×10^{-3} μg/ml for the B and C subtype clones, respectively, a 3-to-6 order of magnitude improvement in the anti-HIV-1 activity (Table 1) based on standard evaluations for compounds in combination [11,31,32].

Cinnamon and green tea extracts possess anti-HIV-1 activities

As found for the elderberry extract, chemical characterization using DART TOF MS (Figure 6A and 6B, respectively) revealed a great variety of chemical species in the cinnamon and the green tea extracts (>200 for cinnamon and >1,100 for green tea). The direct binding assay also showed that TMQ (*m/z* $[M+H]^+$=359.324 AMU) was present in both the cinnamon and green tea extracts. In addition, the presence of the A-type PAC at *m/z* $[M+H]^+$=607.552 AMU in the cinnamon extract was revealed from DART TOF MS analysis (Figure 7A) and the direct binding assay with HIV-1 virus particles. The cinnamon extract possessed a 6:1 ratio of TMQ to the A-type PAC and showed an identical virion-bound ratio, which is consistent with the higher HIV-1 entry inhibition activity observed for cinnamon compared with elderberry. The green tea extract was equally as active as the cinnamon extract partly because of the abundance of EGCG. Entry inhibition and infection was mediated by TMQ binding to the virus and EGCG binding to the CD4 receptors [18]. The green tea does not contain the A-type PAC, and therefore, it was not detected among the compounds bound to the HIV-1 virions (Figure 7B).

The foci reduction assays on the cinnamon and green tea extracts showed dose-dependent inhibition of HIV-1 infection with IC_{50} values lower than those determined for the elderberry extract (Table 1). The cinnamon extract possessed IC_{50} values of 0.7–33 μg/ml and IC_{100} values of 12–106 μg/ml,whereas the green tea extract showed IC_{50} values (0.5–5.4 μg/ml)

Figure 5. Post-direct binding inhibition of infection



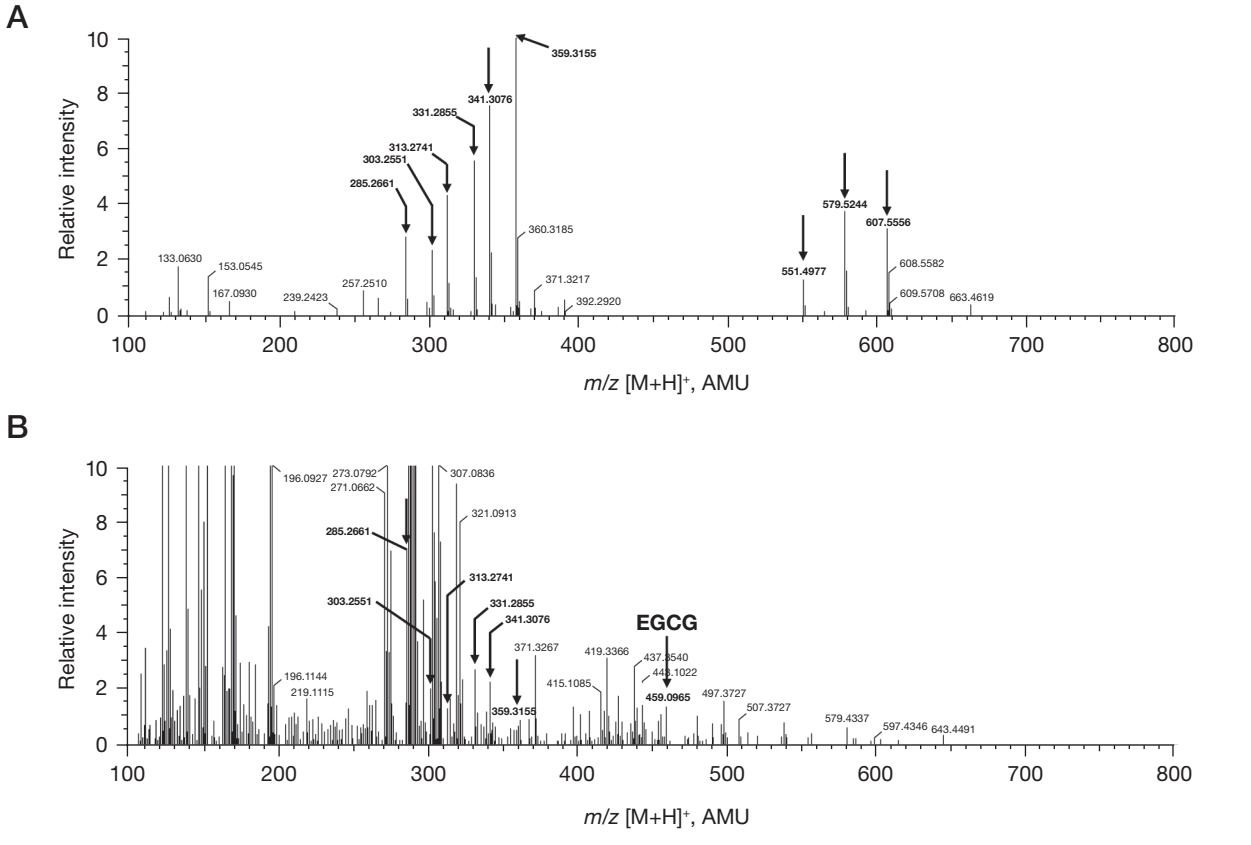
Inhibition of HIV type-1 (HIV-1) after incubating the virus particles in elderberry, cinnamon and green tea extracts. Inhibition of HIV-1 infection was achieved after incubating the virions in various botanical extracts at their 50% inhibitory concentration (IC_{50}) and 100% inhibitory concentration (IC_{100}; see Table 1) for 1 h and washing the unbound chemistries through an Amicon 100 kDa membrane filter (Millipore, Billerica, MA, USA). The virus particles with the bound chemistries were collected and analysed in an HIV-1 infection assay as described in the Methods section.

and IC_{100} values (17–108 µg/ml) similar to cinnamon, but with slightly different efficacies against the different HIV-1 subtypes (Table 1). Although the green tea extract lacked the A-type PAC, the close similarity of the IC_{50} values for HIV-1 infection inhibition of the cinnamon and green tea extracts (Table 1) indicates that the A-type PAC contributes to, but might not be required for, the anti-HIV-1 activities observed here.

EGCG plays a lesser role than virion-bound compounds in HIV-1 infection inhibition

Recent findings by Williamson *et al.* [18] showed that EGCG, found in green tea, inhibits HIV-1 infection by binding to CD4 receptors. We sought, therefore, to separate the HIV-1 inhibitory effects of EGCG (*m/z* $[M+H]^+$=459.100 AMU; Figure 6B) from the entry inhibition activities of TMQ in the green tea extract. GHOST cells were incubated with the green tea extract in order to bind and/or adsorb the majority of EGCG and any other chemicals that might bind to the GHOST cells and their receptors. The supernatant, depleted in chemicals bound to GHOST cells and termed 'depleted

Figure 6. Chemical characterization of the cinnamon and green tea extracts



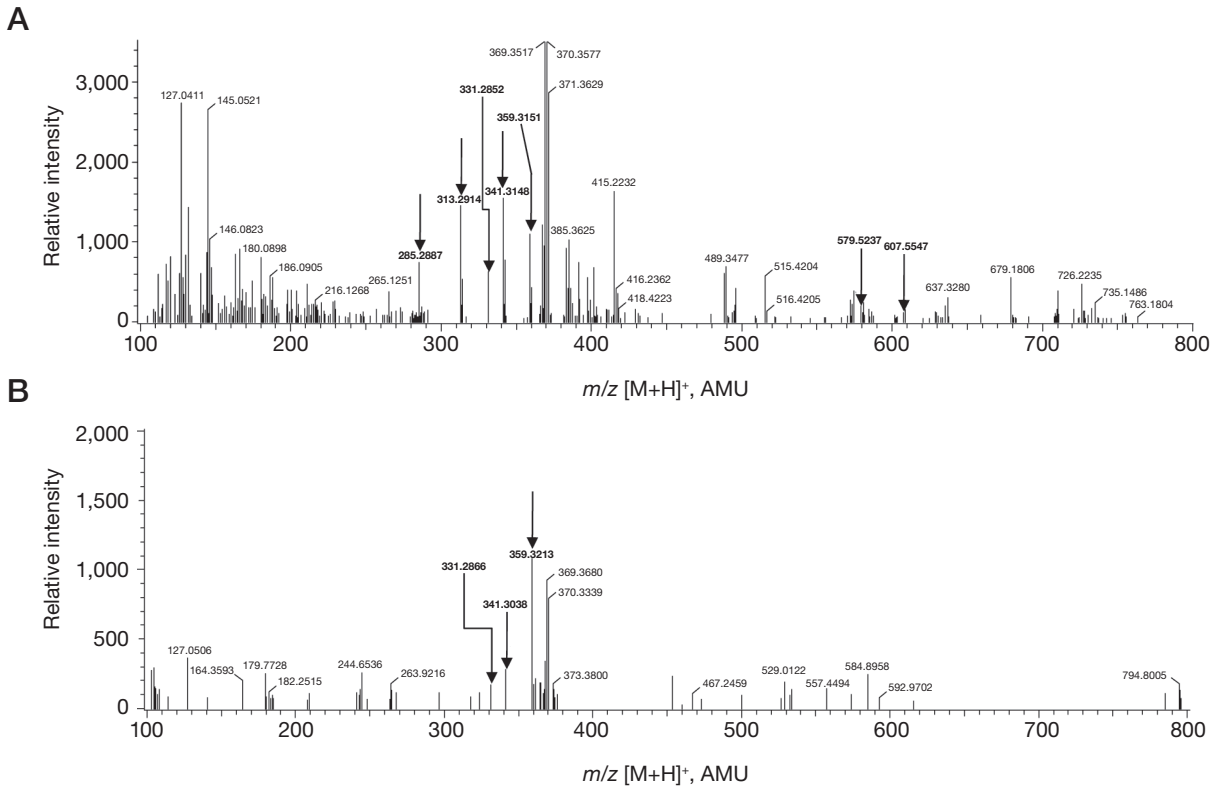
Positive ion $[M+H]^+$ direct analysis in real-time time-of-flight mass spectrometry fingerprints of **(A)** cinnamon and **(B)** Japanese green tea extracts. EGCG, epigallocatechin gallate. AMU, atomic mass units.

extract', was then used in foci reduction assays. The HIV-1 infection inhibition activity (on the basis of IC_{50} and IC_{100} values) obtained from the depleted extract was 2–3× less than the whole green tea extract (Table 2). The HIV-1 infection inhibition activity of the pretreated GHOST cells (containing the compounds from the green tea extract that bind/adsorb to GHOST cells) was 26–85× less active than the whole green tea extract (Table 2).

Discussion

The elderberry extract used here was previously shown to be an effective influenza A (H1N1) virus entry inhibitor [19]. This extract is enriched in flavonoids and this class of chemicals has been shown to possess antiviral activity [33,34]. This information, and previous quantitative structure/activity relationship modelling of small molecule anti-adhesion

Figure 7. Cinnamon and green tea extracts direct binding assays



Positive ion $[M+H]^+$ direct analysis in real-time time-of-flight mass spectrometry of compounds from **(A)** the cinnamon extract and **(B)** the Japanese green tea extract that bind to HIV type-1 virions. The virions were incubated for 1 h in the extract, filtered onto a 100 kDa Amicon membrane (Millipore, Billerica, MA, USA) and washed twice with phosphate-buffered saline (pH 7.2) to remove any unbound chemicals. AMU, atomic mass units.

Table 2. Comparison of the IC_{50} and IC_{100} values of Japanese green tea whole extract, the depleted extract and the pretreated GHOST cells

	Whole extract, µg/ml		Depleted extract, µg/ml		Pretreated GHOST cells, µg/ml	
HIV-1 pseudotype	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
11023 (Subtype B1)	0.5	38.3	1.4	75.5	42.7	323.1
11038 (Subtype B2)	5.4	107.5	6.9	71.0	427.5	NA
11312 (Subtype C1)	0.8	17.5	1.6	78.8	25.3	NA
11313 (Subtype C2)	3.0	61.9	6.5	42.4	NA	NA

The depleted extract contained the compounds present in the whole extract that did not bind to GHOST cells. The pretreated GHOST cells were incubated in the whole extract in order to bind/adsorb epigallocatechin gallate and other chemicals in the whole extract that could potentially interact with the GHOST cells and their receptors. HIV-1, HIV type-1; IC_{50}, 50% inhibitory concentration; IC_{100}, 100% inhibitory concentration; NA, not achieved.

bis-phenolic chemicals [20,35], led us to the hypothesis that flavonoids in the extract are active against HIV-1 and that their activity is exerted through binding to HIV-1 virions, thus effectively blocking their ability to infect host cells.

The foci reduction assays showed that the elderberry extract blocks infection of target GHOST cells enriched in CD4 and CCR5 receptors [29] by four different clones of pseudotyped enveloped HIV-1 virions in a dose-dependent fashion. The inhibition of infection observed was caused by the antiviral activity of the extract as such that the MTT toxicity assay clearly showed that the extracts did not have any toxic effect on the GHOST cells at concentrations well above the determined IC_{100} values.

To test the hypothesis that flavonoids present in the elderberry extract bind to HIV-1 virions and thus prevent infection, we utilized a direct binding assay that identifies chemical species bound to viral surfaces [19]. The DART ion source utilizes electronic excited-state species, such as metastable helium and nitrogen atoms, as plasmas. These excited atoms ionize samples directly for analysis in a TOF mass spectrometer [30]. DART TOF MS is capable of analysing surface materials without direct exposure of the samples to increased temperatures and/or electrical potentials as occurs during atmospheric pressure chemical ionization [36] and electrospray ionization [37] mass spectrometric techniques. For these reasons, DART TOF MS is an ideal mass spectrometric system to characterize botanical extracts and is highly suitable for surface analyses. This new method allowed for the detection and identification of compounds from botanical extracts that bind to HIV-1 virions. In addition, the high mass accuracy of the TOF MS permits precise isotope abundance determinations, allowing for the identification of the specific chemical formula and the corresponding structure for a given mass [30]. Verification of the candidate molecular formulae and structures of the HIV-1-bound compounds was accomplished by chemical fragmentation analyses.

The direct binding assay revealed that the first wash fraction from the HIV-1 particles contained nearly all the chemical species present in the whole extracts excluding TMQ, the A-type PAC (when present in the starting extract) and the ions corresponding to the MS-induced fragmentation of these molecules. The two subsequent washes also did not show these compounds, indicating that the binding of these molecules to the viral surface must be very stable. The binding of only two molecules out of the hundreds of diverse chemical entities in multiple extracts supports a functional role for these chemistries, particularly when combined with the previously described data that TMQ in an extract and in pure form was shown to bind to and block influenza A virus (H1N1) entry into and infection of host cells [19]. The binding of TMQ to HIV-1 and H1N1 virions indicates that this compound might be binding to a common structural motif involved in target cell recognition and initial attachment on these two envelope viruses. These findings suggest that divergent viral types might share molecular and structural motifs that are crucial to host cell recognition and entry, providing insight into the mechanistic aspects of the initial infection process.

The other viral-bound flavonoid was determined to most likely be an A-type PAC (*m/z* $[M+H]^+$=607.552 AMU). The assignment of an A-type PAC structure was corroborated by using the DART TOF MS analysis of a PAC B_2 standard. The main difference between B-type and A-type PACs is the number and types of bonds between the flavonoid subunits (one C–C bond in B-type and one C–C plus one C–O bond in A-type PACs) [38], which renders the A-type PACs generally more stable, supporting the assignment of the *m/z* $[M+H]^+$=607.552 AMU chemical species found bound to the HIV-1 virions as an A-type PAC. Mass spectrometric fragment analysis also supports the proposed A-type PAC identification. Lastly, PAC B_2 yielded HIV-1 infection inhibition IC_{50} values, which were 2–3× less effective than the elderberry extract, whereas IC_{100} values were never achieved. These findings are in full agreement with previous work that showed that A-type PACs are more effective inhibitors of HIV-1 infection than B-type PACs [21]. Confirmation that the binding of these two compounds does indeed inhibit viral infection was provided by post-direct binding infection investigations. In fact, the HIV-1 particles incubated in the IC_{50} and IC_{100} concentrations of elderberry, cinnamon or green tea extract followed by foci reduction assays after removing the unbound compounds, showed IC_{50} and IC_{100} values similar to those obtained from the whole extract indicating that the compounds that bind to HIV-1 virions inhibit viral infection in a stoichiometric fashion.

The direct binding assay not only revealed that TMQ and the A-type PAC bind to the HIV-1 particles and function to block viral entry and infection of GHOST cells, but that the binding ratio of these compounds was dramatically different from their ratios in the extracts. The HIV-1 envelope is composed of two lipid layers and several copies of a glycoprotein complex composed of three gp120 and three gp41 monomers. These HIV-1 glycoprotein complexes are the viral surface components that recognize CD4 and/or CCR5 receptors [39]. The molecules identified here might interact with the lipid bilayer or with a part of the glycoprotein complex to prevent the viral entry to host cells. If TMQ and the A-type PAC bound to the HIV-1 viral lipid bilayer, a specific binding stoichiometry would not be expected

as binding would be dictated primarily by mass action. However, the 6:1 (cinnamon) and 12:1 (elderberry) binding ratios of TMQ:A-type PAC observed here supports the hypothesis that TMQ and the PAC interact with defined components of the envelope glycoprotein complexes and have little to no interaction with the viral lipid bilayer.

To test this hypothesis and to ascertain a possible glycoprotein binding site, we utilized an interaction matrix approach to examine the possible inhibitory, additive or synergistic interactions between the known HIV-1 entry inhibitor enfuvirtide and the elderberry extract. Enfuvirtide is known to bind to the HIV-1 gp41 glycoproteins preventing the gp41 zipping mechanism and blocking membrane fusion [5]. When enfuvirtide was combined with the elderberry extract, a synergy of nearly 6 orders of magnitude was observed. This indicates that the active antiviral chemistries in the elderberry extract are not competing with enfuvirtide binding to gp41 and, therefore, they are most likely acting on gp120 complexes.

Previous studies have shown that cinnamon and green teas are rich in flavonoids and polyphenolics [40,41] and, therefore, we hypothesized that extracts of cinnamon and green tea should also possess compounds that function as HIV-1 entry inhibitors. The cinnamon and green tea extracts examined here showed dose-dependent inhibition of HIV-1 infection with IC_{50} values lower than those determined for elderberry. The mass spectrometric analysis of these extracts revealed that TMQ was also present in green tea and cinnamon, and that TMQ bound to HIV-1 virions effectively blocking viral entry into GHOST cells. These findings further support the antiviral function of TMQ and its anti-entry mode of action.

Although the absence of the A-type PAC in the green tea extract does not correlate with a decrease in the HIV-1 inhibition activity, the A-type PAC cannot be required for the anti-HIV-1 activities observed here, but might contribute to the activity when present. The greater anti-HIV-1 activity of the cinnamon extract compared with elderberry correlates well with the greater relative abundances of both TMQ and the A-type PAC in the cinnamon extract and a lower ratio of TMQ to PAC in the cinnamon extract (6:1) compared with the elderberry extract (12:1).

A recent human pharmacokinetic study conducted on the elderberry extract utilized here revealed that TMQ and the A-type PAC were rapidly taken up into the bloodstream and possessed high bioavailability compared with other flavonoids and polyphenolics [42]. Both compounds had significant residence times in serum and were excreted in the urine.

Recently, EGCG was found to bind to CD4 receptors and inhibit HIV-1 infection [18]. This finding led us to examine whether EGCG binding to CD4 receptors was a more effective inhibitor of HIV-1 infection than TMQ binding to HIV-1 virions. It was found that green tea extracts depleted of EGCG and other compounds that bind to the GHOST cell viral receptors were much more active in blocking infection than the whole green tea extract. These results showed that the compounds present in the green tea extract that bind to the HIV-1 virions are the primary components affecting the HIV-1 inhibition activity of the extract and that only a minimal secondary effect is observed as a result of extract interactions with the GHOST cell receptors (for example, CD4 receptors).

In conclusion, through the use of a newly developed direct binding assay combined with DART TOF MS, we were able to identify and characterize flavonoids from elderberry, green tea and cinnamon extracts that bind to HIV-1 virions and, in so doing, block the infection of host cells likely through the CD4/CCR5 receptor system. Furthermore, these compounds work synergistically with the fusion inhibitor enfuvirtide, which targets the HIV-1 gp41 glycoprotein, indicating that the virion-bound flavonoids are likely to bind to HIV-1 gp120 glycoproteins. On the basis that gp120 forms trimeric structures on the surface of HIV-1 virions [43], glycan-binding chemicals target gp120 [44], mannose-rich regions surround the CD4-binding domains of gp120 [45] and the strong synergy of the elderberry extract with enfuvirtide observed here, we propose that the anti-HIV-1 compounds in the extract most likely bind to the HIV-1 gp120 glycoproteins.

As a result, these flavonoids are potential small molecule lead compounds for new anti-HIV-1 drugs addressing a new therapeutic target for HIV-1/AIDS, and the identified compounds and botanical extracts show promise for cotherapy uses with existing HIV-1 antiviral agents. In addition, the direct binding assay, coupled with the DART TOF MS provides a unique approach to discovery and identification of compounds in botanical extracts that inhibit the infection of HIV-1, and potentially other viruses, through binding to virus particles.

Acknowledgements

The authors thank Dan Li for providing the botanical extracts and Matthew D McMichael for helpful discussions. RCF, BR and RSA conceived and designed the experiments. RCF and BR performed the experiments. RCF, BR and RSA analysed data. RCF, BR and RSA wrote the paper.

Disclosure statement

The authors declare no competing interests.

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Received 8 December 2008, accepted 1 April 2009