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Evaluation of the RNase H Inhibitory Properties of Vietnamese Medicinal Plant Extracts and Natural Compounds

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Abstract

In research on anti-human immunodeficiency virus (HIV) agents from natural sources, thirty two extracts of Vietnamese plants and twenty five isolated compounds were screened for their inhibitory effect against the ribonuclease H (RNase H) activity of HIV-1 reverse transcriptase and the cytopathic effect of the HIV virus. At a concentration of 50 μg/mL, eleven plant extracts and five isolated compounds inhibited over 90 percent of RNase H enzymatic activity. Of these, the methanol extracts from the leaves of *Phyllanthus reticulatus* and *Aglaia aphanamixis* highly inhibited RNase H activity by 99% and 98%, respectively. Several fucoidans isolated from seaweeds *Sargassum kuetzingii*, *Sargassum polycystum*, and *Gelidiella acerosa*, as well as epigallocatechin-3-gallate isolated from *Camellia chinensis* also showed strong inhibitory effects over ninety percent. Sixteen plant extracts with inhibition of over seventy five percent in the RNase H assay were tested in a cellular model of HIV-1 cytopathicity; four extracts showed modest activity in protecting against the cytopathic effect of the HIV virus.

Keywords

RNase H; Cytopathic; Antivirus; Vietnamese plants; Screening

Introduction

The human immunodeficiency virus (HIV) is a pathogenic retrovirus and the cause of acquired immune deficiency syndrome (AIDS). The first cases of AIDS were identified in the United States in 1981. After that, AIDS explosively developed and rapidly became an epidemic disease worldwide. Currently, the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that 33.4 million people around the world are suffering from HIV/AIDS. The annual number of new HIV infections declined from about 3.0 million in 2001 to 2.7 million in 2008 (UNAIDS, 2009). In Asia, reported national HIV prevalence is the

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highest in the southeastern countries, with wide variation in epidemic trends between countries. While the epidemics in Cambodia, Myanmar, and Thailand all show recent declines in HIV prevalence, those in Indonesia and Vietnam are growing. The estimated number of people living with HIV in Vietnam has more than doubled between 2000 and 2005 from 120,000 to 260,000 (UNAIDS, 2009). However, the available antiretroviral drugs at present have fallen short of expectations in many ways. There is the problem of rapid development of resistance of the HIV virus to the drugs, thereby making them inaccessible to those who badly need them in the developing countries.

Reverse transcriptase (RT) of HIV has been demonstrated to be important for the HIV life cycle, which has made it one of the most reliable targets for potential anti-AIDS chemotherapy. This enzyme is a multifunctional enzyme exhibiting not only reverse transcript RNA-dependent DNA polymerase (RDDP) activity but also DNA-dependent DNA polymerase (DDDP) and inherent ribonuclease H (RNase H) activities. The DNA polymerizing functions, together with the RNase H function, are responsible for converting the viral genomic RNA into proviral double-strand DNA. Inhibition the catalytic functions of RT interferes with virus production. Inhibitors of RT have been classified in two broad groups, nucleoside analogues and non-nucleoside inhibitors, with different inhibitory mechanisms, and both types have been found useful as therapeutics (Cruchaga *et al.*, 2007). Unfortunately, their use for treatment of AIDS patients is limited due to the emergence of viral cross-resistance and cellular toxicity. Therefore the development of specific and potent antiviral drugs to restrain infection by HIV-1 still remains an urgent need.

Herein, we report on the investigation of thirty two extracts and twenty five natural compounds from Vietnamese plants for their inhibitory effects against HIV-1 RNase H activity and their activity in reversing the cytopathic effect of the HIV virus.

Material and methods

Materials and preparation of samples—All plants were collected in the different geographical zones of Vietnam. The plants were botanically identified by Prof. Ngo Van Trai, at the National Institute of Medicinal Materials (NIMM), Ministry of Health, Hanoi, Vietnam. Voucher specimens were deposited in the herbarium of the NIMM. The collected plant samples were dried in the shade and ground to a powder. Twenty grams of each dried plant sample was ultrasonically extracted in 100 ml of different solvents at room temperature for three times and filtered. The filtered solutions were combined and evaporated *in vacuo* to give the plants extracts. Natural compounds were obtained from several plants as previously published (Cuong *et al.*, 2006; Cuong and Tuan, 2006; Nhut *et al.*, 2007; Cuong *et al.*, 2010b). The plant extracts and natural products were dissolved in dimethyl sulfoxide (DMSO) for bioassay.

Reagents—The oligonucleotides 5′-GAU CUG AGC CUG GGA GCU-fluorescein-3′ and 5′-Dabcyl-AGC TCC CAG GCT CAG ATC-3′ were synthesized and provided as the annealed RNA/DNA hybrid by TriLink Biotechnologies (San Diego, CA). The oligonucleotides 5′-$(rA)_{22}$-fluorescein-3′ and 5′-Dabcyl-$(dT)_{22}$-3′ were products of Dharmacon (Lafayette, CO) and Midland Certified Reagent Co. (Midland, TX), respectively. The hybrid heteroduplex was formed by mixing 5′-$(rA)_{22}$-fluorescein-3′ and 5′-Dabcyl-$(dT)_{22}$-3′ dissolved in 50 mM Tris, pH 8.0, containing 60 mM KCl, in a ratio of 1:1.2 followed by heating at 90°C for 5 min and slow cooling to room temperature. Aliquots of stock hybrid homoduplex were stored at −20 °C until use. Recombinant wild-type p66/p51 HIV-1 RT was overexpressed and purified as described (Fletcher *et al.*, 1996).

Biological Testing

Spectroscopic measurements—Details of the RNase H FRET assay have been previously described (Parniak *et al.*, 2003). Inhibition assays in 96-well microplates were carried out using a SpectraMax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). Assays in 384-well microplates were performed using a Victor^{2}V multilabel plate reader (Perkin–Elmer Life Sciences, Boston, MA).

Microplate assay of RNase H activity—Assays were carried out in a total volume of 100 μL of 50 mM Tris, pH 8.0, containing 60 mM KCl and 5 mM $MgCl_2$, using final concentrations of 0.25 μM RNA/DNA hybrid and 1.0 nM recombinant p66/p51 HIV-1 RT. Stock solutions of the RNA/DNA hybrid and HIV-1 RT were diluted to the appropriate concentration immediately before use.

Reactions were started by the addition of HIV-1 RT and allowed to proceed at 37 °C for 30 min. Reactions were quenched by the addition of 50 μL of 0.5 M EDTA, pH 8.0. Fluorescence intensity in each well was assessed using an excitation wavelength of 490 nm and an emission wavelength of 528 nm, with cutoff filter set to 515 nm. To assess the effect of inhibitors, 1 μL of inhibitor in DMSO was added to the microplate well prior to the addition of substrate and RT solutions (Parniak *et al.*, 2003)

HIV-1 cytopathicity assay—Samples were tested in duplicate dose response format using HIV-1_{RF} in CEM-SS cells by a previously published method (Weislow *et al.*, 1989). Extracts were dissolved in DMSO at 20 mg/mL, and diluted 1:200 into the assay plates, yielding a final top concentration of 100 μg/mL, with eight 2-fold dilutions to a low dose of 0.78 μg/mL.

Statistical Analysis—Statistical analysis was performed using the spreadsheet program Excel (Microsoft Office 2007). The data are the mean of three repeated experiments in triplicate. Values varied by no more than 5% between experiments.

Results and discussion

In the aim of our studies to discover active anti-HIV substances, 32 plants extracts and 25 compounds from natural sources were screened for RNase H enzymatic inhibition. The RNase H inhibition was measured using a FRET assay which has been previously described (Parniak *et al.*, 2003). Using this method, the Vietnamese medicinal plants screened for RNase H inhibition are shown in Table 1. Among samples examined, the extracts of *Aglaia aphanamixis*, *Bousingonia mekongense*, *Camellia chinensis*, *Eurya annamensis*, *Eurya ciliata*, *Fissistigma polyanthoides*, *Goniothalamus gracillipes*, and *Phyllanthus reticulatus* showed over 90 % inhibitory effect on RNase H at the concentration of 50 μg/mL. The remaining samples exhibited moderate but significant inhibitory activity (56–89%). In the highly inhibitory plants, *A. aphanamixis*, *B. mekongense*, *C. chinensis*, and *G. gracillipes* have not previously been reported in either phytochemical or biological studies. *E. ciliata and E. annamensis* have been found to contain triterpene fatty acid esters and flavonoids such as apigenin, chrysoeriol, and quecitrin which were found to exhibit considerable monoamine oxygenase (MAO) inhibitory activity (Cuong *et al.*, 2006; Cuong and Tuan, 2006). Chrysoeriol from *E. ciliata* also was found to enhance the proliferation and differentiation of osteoblastic MC3T3-E1 cells (Tai *et al.*, 2009). In considering effect of these plants on the anti-HIV screening, 50 μg/mL of the methanol extracts of leaves and stem of *P. reticulatus* were the most active against RNase H, with inhibition of 99% and 96 %, respectively. In folk remedies, *P. reticulatus* is used for a variety of ailments including smallpox, syphilis, asthma, diarrhoea, and bleeding from gums. It also is claimed to have

anti-diabetic activity in tribal areas (Kumar *et al.*, 2008). *Phyllanthus* species have been found to contain bioactive alkaloids, flavonoids, lignan, phenol and terpenes (Lam *et al.*, 2007). Some reports have demonstrated its anti-plasmodial activity (Omulokoli *et al.*, 1997) and antidiabetic activity (Kumar *et al.*, 2008). However, there are no reports on its inhibitory effect against RNase H.

To further evaluate the plant extracts, those which inhibited RNase H in the enzymatic assay by >75 percent were tested in a cellular model of HIV-1 cytopathicity. Dose-response curves of each sample were constructed at eight different concentrations in the range of 0.78–100 μg/mL to obtained EC_{50} and IC_{50} values. Of the 16 extracts tested, only four showed significantly protection against the viral cytopathic effect. These were the BuOH extract of *Celastrus orbiculata* leaves, and the MeOH extracts of *Glycosmis stenocarpa* stems, *E. ciliata* leaves, and *P. reticulatus* leaves. (Table 1).

Similarly, twenty five isolated or semi-synthetic compounds from natural sources were also tested in the enzymatic assay. At the concentration of 50 μg/mL, almost half of these samples showed potent inhibition of activity with a range of 49–97% (table 2). Of these, several fucoidan compounds, epigallocatechin-3-gallate (EGCG), and indirubin-3′-oxime exhibited higher effect with inhibition values over 80% at the tested concentration. EGCG is the most abundant catechin in green tea, and is also a potent antioxidant that may have therapeutic properties for many disorders including cancer (Katiyar *et al.*, 2007). There has been research investigating the benefit of EGCG from green tea in the treatment of HIV infection. It has been shown to reduce plaques related to AIDS-related dementia as well as to block gp120. More research on EGCG and HIV is currently underway (Williamson *et al.*, 2006). In considering effects on RNase H activity, EGCG exhibited strong inhibition and it may be the major compound causing high activity of green tea extract, as well as *C. chinensis*. The fucoidan compounds isolated from seaweeds also showed strong activity. Pharmaceutical research has been done on fucoidans, which are now being marketed as nutraceuticals and food supplements. Others reports indicated that fucoidan compounds can induce apoptosis in human lymphoma cell lines, and inhibit hyperplasia in rabbits (Aisa *et al.*, 2004). Finally, the active compound indirubin-3′-oxime is now considered as new class of compound for treatment of cancer, especially leukemia and other immunological diseases (Kagialis-Girard *et al.*, 2007). Indirubin-3'-oxime was synthesized by a condensation reaction between hydroxylamine and indirubin. Indirubin can be easily prepared from the leaves of several plants, such as *Polygonum tinctorium* (Polygonaceae), *Isatis indigotica* (Bassicaceae), *Indigofera suffrutticosa* (Fabaceae), *Indigofera tinctoria* (Fabaceae) and *Strobilanthes cusia* (Acanthaceae) (Cuong *et al.*, 2010a; Cuong *et al.*, 2010b). Recently, indirubin-3′-oxime was found to induce cell cycle arrest and apoptosis in Hep-2 human laryngeal carcinoma cells (Kameswaran and Ramanibai, 2009). In the effects on RNase H activity, indirubin-3′-oxime exhibited inhibition of 82% at the concentration of 50 μg/mL. Based on this result, indirubin-3′-oxime and its derivatives may have potential to be developed as cancer drugs as well as for antiviral disease.

Conclusion

Fifty seven plant samples including extracts of Vietnamese plants and natural compounds were screened for their inhibitory effect against the ribonuclease H (RNase H) activity of HIV-1 reverse transcriptase. Of those, at the concentration of 50 μg/mL, methanol extracts from the leaves of *P. reticulatus* and *A. aphanamixis* demonstrated the strongest RNase H activity (99% and 98%, respectively). The extracts of *B. mekongense*, *C. chinensis*, *E. annamensis*, *E. ciliata*, *G. gracillipes*, and *F. polyanthoides* also showed highly inhibitory effects against RNase H activity with inhibition values of more than 90%. Natural compounds such as epigallocatechin-3-gallate isolated from *C. chinensis*, fucoidans

separated from seaweeds *Sargassum kuetzingii*, *Sargassum polycystum*, *Gelidiella acerosa*, and indirubin-3′-oxime inhibited RNase H activity with the inhibition values over 90 percent. Four of the plant extracts had moderate activity in an HIV-1 cytopathicity assay.

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Table 1

RNase H inhibitory effect of Vietnamese medicinal plant extracts

No.	Botanical name	Codes	Family name	Part used(*)	Extract	RNase H inhibition (% at 50 μg/mL)	HIV-1 EC_{50} (μg/mL)	HIV-1 IC_{50} (μg./mL)
1	*Aglaia aphanamixis*	VHKC-0063	Meliaceae	L	MeOH	98	n.p.	8.9
2	*Bousingonia mekongense*	VHKC-0049	Apocynaceae	WP	$CHCl_3$	97	n.p.	3.9
3	*Bousingonia mekongense*	VHKC-0048	Apocynaceae	WP	*n*-Hexane	17	---	---
4	*Buddlejia officinalis*	VHKC-0058	Loganiaceae	F	EtOAc	63	---	---
5	*Buddlejia officinalis*	VHKC-0011	Loganiaceae	F	Water	32	---	---
6	*Buddlejia officinalis*	VHKC-0012	Loganiaceae	F	MeOH	2	---	---
7	*Camelia chinensis*	VHKC-0019	Theaceae	L	EtOAC	90	n.p.	2.1
8	*Celastrus orbiculata*	VHKC-0043	Celastraceae	L	BuOH	78	44.9	>100
9	*Celastrus orbiculata*	VHKC-0042	Celastraceae	L	EtOAc	89	n.p.	33.0
10	*Eurya annamensis*	VHKC-0062	Theaceae	L	MeOH	93	n.p.	7.6
11	*Eurya ciliata*	VHKC-0054	Theaceae	L	MeOH	96	12.1	15.9
12	*Fissistigma polyanthoides*	VHKC-0052	Annonaceae	L	MeOH	95	n.p.	17.8
13	*Fissistigma polyanthoides*	VHKC-0053	Annonaceae	SB	MeOH	95	n.p.	28.1
14	*Glycosmis stenocarpa*	VHKC-0057	Rutaceae	S	MeOH	77	7.1	16.3
15	*Goniothalamus gracillipes*	VHKC-0039	Annonaceae	L	*n*-Hexane	56	---	---
16	*Goniothalamus gracillipes*	VHKC-0040	Annonaceae	L	$CHCl_3$	79	n.p.	3.4
17	*Goniothalamus gracillipes*	VHKC-0041	Annonaceae	L	EtOH	97	n.p.	3.5
18	*Goniothalamus tamirensis*	VHKC-0037	Annonaceae	L	$CHCl_3$	66	---	---
19	*Goniothalamus tamirensis*	VHKC-0038	Annonaceae	L	EtOH	47	---	---
20	*Goniothalamus tamirensis*	VHKC-0036	Annonaceae	L	*n*-Hexane	38	---	---
21	*Goniothalamus vietnamensis*	VHKC-0050	Annonaceae	L	MeOH	81	n.p.	11.7
22	*Goniothalamus vietnamensis*	VHKC-0051	Annonaceae	R	MeOH	17	---	---
23	*Panax stipuleanatus*	VHKC-0044	Araliaceae	Rh	MeOH	31	---	---
24	*Peristrophe roxburghiana*	VHKC-0013	Acanthaceae	L	Water	6	---	---
25	*Phyllanthus reticulatus*	VHKC-0055	Euphorbiaceae	L	MeOH	99	5.6	6.3
26	*Phyllanthus reticulatus*	VHKC-0056	Euphorbiaceae	S	MeOH	96	n.p.	20.8
27	*Schefflera leucantha*	VHKC-0045	Araliaceae	L	CH_2Cl_2	66	---	---
28	*Strobilanthes cusia*	VHKC-0016	Acanthaceae	L	CH_2Cl_2	67	---	---

No.	Botanical name	Codes	Family name	Part used(*)	Extract	RNase H inhibition (% at 50 μg/mL)	HIV-1 EC_{50} (μg/mL)	HIV-1 IC_{50} (μg./mL)
29	*Strobilanthes cusia*	VHKC-0017	Acanthaceae	L	EtOAc	92	n.p.	17.1
30	*Strobilanthes cusia*	VHKC-0018	Acanthaceae	L	BuOH	42	---	---
31	*Trichosanthes kirilowii*	VHKC-0047	Cucurbitaceae	R	Water	8	---	---
32	*Trichosanthes kirilowii*	VHKC-0046	Cucurbitaceae	R	CH_2Cl_2	13	---	---

(*)B: bark, F: flowers, L: leaves, R: root, Rh: rhizome, S: stem, SB: stem bark, WP: whole plant; n.p. no protection from the cytopathic effect of the virus (inactive)

Table 2

RNase H inhibitory effect of some natural compounds

No.	Product name	Sources		RNase H inhibition (%)
		Botanical name	Family name	
1	Chalcon	*Carya tonkinensis*	Juglandaceae	12
2	Chrysoeriol	*Eurya cilliata*	Theaceae	49
3	Epigallocatechin-3-gallate	*Camelia chinensis*	Theaceae	93
4	Fucoidan	*Sargassum kuetzingii*	Sargassaceae	96
5	Fucoidan	*Ulva reticulata*	Ulvaceae	32
6	Fucoidan	*Gracilaria fisheri*	Gracilariaceae	77
7	Fucoidan	*Gracilaria firma*	Gracilariaceae	26
8	Fucoidan	*Ulva fenestrata*	Ulvaceae	49
9	Fucoidan	*Gracilaria tenuistipitata*	Gracilariaceae	21
10	Fucoidan	*Sargassum xuanmaii*	Sargassaceae	85
11	Fucoidan	*Gracilaria bailimiae*	Gracilariaceae	29
12	Fucoidan	*Gelidiella acerosa*	Gelidiaceae	91
13	Fucoidan	*Gracilaria asiatica*	Gracilariaceae	21
14	Fucoidan	*Sargassum polycystum*	Phaeophyceae	67
15	Fucoidan	*Sargassum polycystum*	Phaeophyceae	92
16	Fucoidan	*Sargassum polycystum*	Phaeophyceae	97
17	Glypetelotine	*Glycosmis petelotii*	Rutaceae	–6
18	1-Hydroxy-3-methyl carbazole	*Glycosmis stenocarpa*	Rutaceae	49
19	Indigo naturalis	*Strobilanthes cusia*	Acanthaceae	67
20	Indirubin-3′-oxime	*Strobilanthes cusia*	Acanthaceae	82
21	Linarin	*Buddleja officinalis*	Loganiaceae	2
22	Murrayafoline-A	*Glycosmis stenocarpa*	Rutaceae	42
23	Quercitrin	*Eurya cilliata*	Theaceae	31
24	Sciadopitysin	*Taxus chinensis*	Taxaceae	76
25	Ursolic acid	*Bousingonia mekongense*	Apocynaceae	49