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Differential inhibition of reverse transcriptase and cellular DNA polymerase-α activities by lignans isolated from Chinese herbs, *Phyllanthus myrtifolius* Moon, and tannins from *Lonicera japonica* Thunb and *Castanopsis hystrix*

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Abstract

Two lignans, phyllamycin B and retrojusticidin B isolated from *Phyllanthus myrtifolius* Moon have been demonstrated to have a strong inhibitory effect on human immunodeficiency virus-1 reverse transcriptase activity (HIV-1 RT), but much less inhibitory effect on human DNA polymerase-α (HDNAP-α) activity. Fifty percent inhibitory concentrations of phyllamycin B and retrojusticidin B were determined to be 3.5 and 5.5 μM for HIV-1 RT, and 289 and 989 μM for HDNAP-α, respectively. The mode of inhibition was found to be non-competitive inhibition with respect to template-primer and triphosphate substrate. Several tannins such as caffeoylquinates (CQs) isolated from *Lonicera japonica* Thunb, galloylquinates (GQs) and galloylshikimates (GSs) purified from *Castanopsis hystrix* were shown to have a much less selective inhibitory effect on HIV-1 RT.

Keywords: Lignan; Caffeoylquinate; Galloylquinate; Galloylshikimate; Reverse transcriptase inhibition

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1. Introduction

Reverse transcriptase is essential for the replication of human immunodeficiency virus. Many attempts have been made to develop reverse transcriptase inhibitors, and only a few compounds were found to be clinically useful to date. The nucleoside analog, 3′-azido-3′-deoxythymidine (AZT), currently in clinical use, acts by its active metabolite, AZT-TP, inhibitory reverse transcriptase activity by acting as a chain terminator (Mitsuya et al., 1985; Parker et al., 1991). A second nucleoside analog, 2′,3′-dideoxyinosine (ddI), was approved for clinical use (Ahluwalin et al., 1987). Chinese herbs represent an important potential source of reverse transciptase (RT) inhibitors. Several tannins isolated from Chinese galls, were found to have a strong inhibitory effect against human immunodeficiency virus 1 reverse transcriptase (HIV-1 RT) (Nishizawa et al., 1989; Nonaka et al., 1990; Chang et al., 1994). Various flavonoids were shown to inhibit RT of certain retroviruses including HIV-1 or Moloney murine leukemia virus RT activities (Ono et al., 1990; Chu et al., 1992). Five tetrahydroxyxanthones, isolated from *Tripterospermum lanceolatum* (Hyata) were demonstrated to have a strong inhibitory effect on Moloney murine leukemia virus RT activity (Chang et al., 1992).

Phyllanthus myrtifolius Moon (Euphorbiaceae) is widely grown in southern China. In the present study, the anti-HIV-1 RT activity of lignans isolated from *P. myrtifolius* Moon was demonstrated and compared with that of tannins, caffeoylquinates (CQs), isolated from *Lonicera japonica* Thunb, and galloylquinates (GQs) and galloylshikimates (GSs) from *Castanopsis hystrix*.

2. Materials and methods

2.1. Chemicals

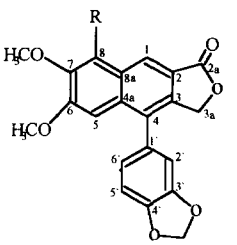
(Riboadenylic acid)$_n$-(deoxythymidylic acid)$_{15}$, and activated calf thymus DNA were obtained from Pharmacia, Uppsala, Sweden. HIV-1 RT was purchased from HT Biotechnology Ltd., Cambridge, UK. Human DNA polymerase-α (HDNAP-α) was purified from human placenta (Matsukage et al., 1976; Syväoja et al., 1990). CQs were isolated from *L. japonica* Thunb (Chang, 1990). GSs and GQs were purified from *C. hystrix* (Ishimaru et al., 1987). Six lignans, phyllamycin A, phyllamycin B, phyllamycin C, retrojusticidin B, justicidin A and justicidin B were isolated from *P. myrtifolius* Moon (Euphorbiaceae) (Lin et al., 1994). For structures of lignans and CQ and GQ see Fig. 1.

2.2. Reverse transcriptase assay

The compounds to be analyzed were prepared as 1 mM stock solution in 50% DMSO. HIV-1 RT was diluted in 50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 0.1% Nonidet-P40, 2 mM β-mercaptoethanol, 50% (v/v) glycerol and stored at -20°C. Experiments were performed at 37°C using an assay mixture (25 μl) consisting of 50 mM Tris-HCl, pH 8.5, 80 mM KCl, 10 mM dithiothreitol, 6 mM $MgCl_2$, 0.1%

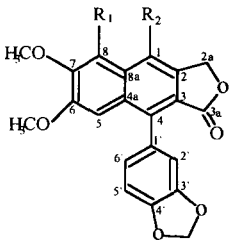
Lignans

A



1 R=OCH3

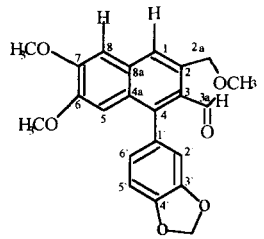
4 R=H



3 R_1=OCH_3, R_2=H

5 R_1=H, R_2=OCH_3

6 R_1=H, R_2=H

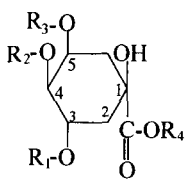


2

1. Phyllamycin A
2. Phyllamycin B
3. Phyllamycin C
4. Retrojusticidin B
5. Justicidin A
6. Justicidin B

Caffeoylquinate

B



Monocaffeoylquinate	R_1	R_2	R_3	R_4
Chlorogenic acid	Caf	H	H	H
Dicaffeolquinate				
3.4-di-O-caffeoylquinic acid	Caf	Caf	H	H
3.5-di-O-caffeoylquinic acid	Caf	H	Caf	H
4.5-di-O-caffeoylquinic acid	H	Caf	Caf	H
Methyl 3.4-di-O-caffeoylquinic acid	Caf	Caf	H	CH_3
Methyl 3.5-di-O-caffeoylquinic acid	Caf	H	Caf	CH_3

Caf:Caffeoyl group

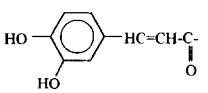
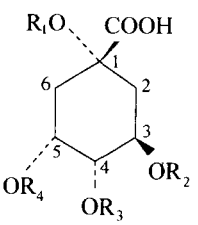


Fig. 1. Structure formulae of isolated lignans from *Phyllanthus myrtifolius* Moon, and various tannins from *Lonicera japonica* Thunb and *Castanopsis hystrix*.

Galloylquinic acid C



	R1	R2	R3	R4
3-O-galloylquinic acid	H	G	H	H
5-O-galloylquinic acid	H	H	G	H
3.4-di-O-galloylquinic acid	H	G	G	H
3.5-di-O-galloylquinic acid	H	G	H	G
3.4.5-tri-O-galloylquinic acid	H	G	G	G

Fig. 1 (continued).

diethylpyrocarbonate, 3 μg nuclease free bovine serum albumin, 0.5 μg synthetic template-primer poly(rA)$_n$-oligo (dT)$_{15}$, 1.5 μl of 0.8 mM dTTP and 0.5 μl of 0.5 μCi/ml [^{3}H]dTTP. Reactions were started by addition of enzyme. After 30 min, the reaction was stopped by addition of 5 ml of trichloroacetic acid and 4% (w/v) sodium pyrophosphate. The samples were cooled at 4°C for 10 min and then 20 μl of reaction mixture was spotted on Whatman DE-81 cellulose paper and washed 5 times with 5% TCA consisting of 2 mM sodium pyrophosphate and two times each with water and absolute alcohol. The filters were air dried and counted in a Beckman 5801 liquid scintillation counter.

For steady state kinetic studies, the concentrations of dTTP were varied from 2.5 to 40 mM, and those of poly(rA)$_n$ oligo(dT)$_{15}$ were between 1 and 16 mg/ml. Reactions were started by addition of enzyme. At the various periods of time, a 25 μl aliquot was removed from the reaction mixture, followed by acid precipitation and washing as described above.

2.3. Human DNA polymerase-α assay

Reaction mixtures (125 μl) were comprised of 20 μl of 1 mg activated calf thymus DNA, 1 μM each of dATP, dGTP and dCTP, 0.5 μCi/ml [^{3}H]dTTP, 10 μg nuclease-free bovine serum albumin, 0.12 unit human DNA polymerase-α, 40 mM Tris-HCl buffer, pH 7.8, 6 mM $MgCl_2$, 2 mM dithiothreitol and various concentrations of compounds to be tested. The reaction was carried out by incubating the mixtures at 37°C for 30 min, and the samples were treated as described above for liquid scintillation counting.

3. Results

3.1. Effects of various lignans and tannins on HIV-1 RT and HDNAP-α

The six lignans, phyllamycin A, phyllamycin B, phyllamycin C, retrojusticidin B, justicidin A and justicidin B isolated from *P. myrtifolius* Moon were examined for their

anti HIV-1 RT activity under the assay conditions as described in Materials and methods. Simultaneously, several tannins, such as 3,5-di-*O*-CQ, 3,4,5-tri-*O*-GQ and 3,4,5-tri-*O*-GS known as anti-AIDS agents (Nishizawa et al., 1989; Nonaka et al., 1990), were also examined and compared. The degree of inhibition of the lignans, phyllamycin A, B, and C, retrojusticidin B, justicidin A and B are 17, 95, 4, 89, 12 and 8%, respectively, at the concentration of 33 μg/ml. The IC_{50} of phyllamycin B and retrojusticidin B were then determined to be 3.5 and 5.5 μM. In order to study whether

Table 1
The IC_{50} of phyllamycin B and retrojusticidin B, and various tannins on HIV-1 RT and HDNAP-α activities

Compounds	IC_{50} (μM)		(B)/(A)
	HIV-RT (A)	DNAP-α (B)	
Lignans			
Phyllamycin B	3.5	289	82.6
Retrojusticidin B	5.49	989	180.1
Caffeoylquinates			
Monocaffeoylquinate			
Chlorogenic acid	– [a]	ND [b]	ND
Dicaffeoylquinate			
3,4-di-*O*-caffeoylquinic acid	19.4	11.6	0.60
3,5-di-*O*-caffeoylquinic acid	1.16	2.32	2.0
4,5-di-*O*-caffeoylquinic acid	349	387	1.11
Methylcaffeoylquinate			
Methyl 3,4-di-*O*-caffeoylquinic acid	94.0	45.0	0.48
Methyl 3,5-di-*O*-caffeoylquinic acid	1.70	3.77	2.22
Galloylquinates			
Monogalloylquinate			
3-*O*-galloylquinic acid	72.6	8.72	0.12
5-*O*-galloylquinic acid	– [a]	ND	ND
Digalloylquinate			
3,4-di-*O*-galloylquinic acid	7.81	0.60	0.08
3,5-di-*O*-galloylquinic acid	1.31	0.48	0.37
Trigalloylquinate			
3,4,5-tri-*O*-galloylquinic acid	0.08	0.17	2.13
Galloylshikimates			
Monogalloylshikimate			
3-*O*-galloylshikimic acid	92.0	12.0	0.13
4-*O*-galloylshikimic acid	– [a]	ND	ND
5-*O*-galloylshikimic acid	– [a]	ND	ND
Digalloylshikimate			
3,4-di-*O*-galloylshikimic acid	1.77	1.05	0.59
3,5-di-*O*-galloylshikimic acid	0.52	0.31	0.60
Trigalloylshikimate			
3,4,5-tri-*O*-galloylshikimic acid	0.10	0.22	2.20

[a] IC_{50} > 333 μg/ml.
[b] ND, not determined.

Table 2
K_i values and the modes of inhibition of lignans and tannins on HIV-1 RT

Compound	Template-primer		dTTP	
	Mode	K_i (μM)	Mode	K_i (μM)
3,4,5-tri-*O*-galloylquinic acid	C [a]	0.04 ± 0.01	NC [b]	0.20 ± 0.01
3,4,5-tri-*O*-galloylshikimic acid	C	0.05 ± 0.01	NC	0.25 ± 0.01
3,5-di-*O*-caffeoylquinic acid	C	1.31 ± 0.23	NC	1.14 ± 0.40
Phyllamycin B	NC	4.31 ± 0.85	NC	4.09 ± 0.40
Retrojusticidin B	NC	4.97 ± 0.31	NC	3.80 ± 0.48

[a] C, competitive.
[b] NC, non-competitive.

the inhibition on HIV-1 RT was specific by phyllamycin B and retrojusticidin B, HDNAP-α was used to examine the inhibitory effects of two lignans. As shown in Table 1, the IC_{50} of phyllamycin B and retrojusticidin B for HDNAP-α is much higher than that for HIV-1 RT, and the ratio of IC_{50} of HDNAP-α and HIV-1 RT is 82.6 and 180.1 for phyllamycin B and retrojusticidin B, respectively.

Of 5 CQs tested, two, 3,5-di-*O*-CQ and methyl 3,5-di-*O*-CQ had a strong inhibitory effect on HIV-1 RT, but they also inhibited HDNAP-α so strongly that the ratio of IC_{50} of HIV-1 RT and HDNAP-α of 3,5-di-*O*-CQ and methyl 3,5-di-*O*-CQ was only 2.0 and 2.2, while 3,4-di-*O*-CQ and methyl 3,4-di-*O*-CQ had higher inhibitory effects on HDNAP-α than HIV-1 RT. This low ratio of IC_{50} for HIV-1 RT and HDNAP-α was also found for several GQs and GSs (Table 2).

3.2. Mode of inhibition and determination of kinetic constants

The inhibition was studied kinetically by changing either the concentration of triphosphate substrate or the template-primer. Phyllamycin B and retrojusticidin B were shown to inhibit HIV-1 RT activity in a non-competitive way with respect to template-primer, $(rA)_n$ $(dT)_{15}$ or triphosphate substrate, dTTP while all CQs, GQs and GSs studied, competitive with respect to template-primer but not with triphosphate substrate. K_i values of all cases were determined and are summarized in Table 2.

4. Discussion

The present investigation demonstrates that phyllamycin B and retrojusticidin B have a higher inhibitory effect on HIV-1 RT activity and a lower inhibitory effect on HDNAP-α activity. Studying the structure and activity relationship, phyllamycin B has a lactone group at position 2a and a hydrogen atom at position 8 which are essential for selective inhibition of HIV-1 RT activity. However, if the lactone ring is open, like retrojusticidin B, the aldehyde group at position 3a is also effective against HIV-1 RT activity. Justicidin A and B, having a keto group of lactone at position 3a and a hydrogen atom at position 8, are not active against HIV-RT activity. Phyllamycin A has

a methoxy group at position 8, not a hydrogen atom is not active against HIV-RT, though there is a keto group of lactone at position 2a. Phyllamycin C, with a methoxy group at position 2, but a keto group of lactone at 3a is not effective against HIV-RT activity.

The results of various tannins showed that they did not have a selective inhibitory effect on HIV-1 RT. It was reported previously that tannins, like a number of polyanionic compounds including dextran sulfate, polyanionic polysaccharide, polyhydroxy carboxylates derived from phenolic compound and flavanoids (Mitsuya et al., 1988; Weiler et al., 1990; Schols et al., 1991; Mahmood et al., 1993) selectively inhibit HIV replication by interacting with the surface glycoprotein gp120 to irreversibly prevent binding of virus to the $CD4^+$ receptor.

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