Antiviral Research, 19 (1992) 119–127

AVR 00574

Inhibition of Moloney murine leukemia virus reverse transcriptase activity by tetrahydroxyxanthones isolated from the Chinese herb, *Tripterospermum lanceolatum* (Hyata)

Chia-Che Chang[a], Chun-Nan Lin[b] and Jung-Yaw Lin[a]

[a]*Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC and* [b]*Department of Pharmacy, Kaoshiung Medical School, Kaoshiung, Taiwan, ROC*

(Received 23 January 1992; accepted 24 April 1992)

Summary

Five tetrahydroxyxanthones (THXs) isolated from *Tripterospermum lanceolatum* (Hyata) have been shown to have a strong inhibitory effect on Moloney murine leukemia virus reverse transcriptase (Mo-MLV RT) activity when (rA)*n*-$(dT)_{15}$ and (rC)*n*-$(dT)_{12-18}$ were used as template-primers. 50% inhibitory concentrations of 1,3,5,6-THX, 2,3,6,7-THX 1,3,6,7-THX, 3,4,5,6-THX, and 3,4,6,7-THX were determined to be 0.15, 0.27, 0.58, 0.12, and 0.12 μM, respectively. Their effects were concentration-dependent, and the mode of inhibition was found to be by competitive inhibition with respect to template-primer. The tetrahydroxyl groups of THXs were shown to be important for their inhibitory activity. Acylation of THXs with various groups resulted in a moderate or strong decrease in their inhibitory activity.

Tetrahydroxyxanthone; Reverse transcriptase; IC_{50}; Enzyme kinetics

Correspondence to: J.Y. Lin, Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1. Jen Ai Rd., Sec. 1, Taipei (10018), Taiwan, ROC.

Abbreviations: THX: Tetrahydroxyxanthone; Mo-MLV RT: Moloney murine leukemia virus reverse transcriptase; DNAP: *E. coli* DNA polymerase I; DMSO: dimethylsulfoxide; EDTA: ethylenediaminetetraacetic acid.

Introduction

Chinese herbs have been traditionally used as remedies in Oriental medicine. Many important biological components have been purified and their biological activities demonstrated. Some plant secondary metabolites, such as saponins and flavonoids (phenylbenzo-γ-pyrones) have been shown to possess anti-RT activity (Apple et al., 1975; Inouye et al., 1989; Kaul et al., 1985; Kakiuchi et al., 1988; Ono et al., 1989; Speddnig et al., 1989; Vrijsen et al., 1987). THXs isolated from the Chinese herb *Tripterospermum lanceolatum* (Hyata) (Lin et al., 1987) were reported to be effective vasorelaxing agent (Ko et al., 1991), and antiplatelet agents (Teng et al., 1989). In this study, the anti-RT activity of THXs was demonstrated and their mechanisms of action were investigated.

Materials and Methods

Chemicals

The THXs used in this study were isolated from *Tripterospermum lanceolatum* (Hyata) (Lin et al., 1987). (Riboadenylic acid)*n*-(deoxythymidylic acid)$_{15}$, (ribocytidylic acid)*n*-(deoxyguanylic acid)$_{12-18}$ and activated calf thymus DNA were purchased from Pharmacia, Uppsala, Sweden. Mo-MLV murine leukemia virus reverse transcriptase was obtained from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD. [^{3}H]dTTP (50 Ci/mmol) and [^{3}H]dGTP (50 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA. DNA polymerase I (DNAP-I) was obtained from Boehringer Mannheim GmbH, Pemzberg, Germany. All other reagents and solvents used were of analytical grade.

Methods

THXs or other compounds to be analyzed were prepared as 1 mM stock solution in 50% DMSO. Mo-MLV RT was diluted in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA. 0.01% (v/v) Nonidet-40 and 50% (v/v) glycerol and stored at -20°C.

Effects of THXs on Mo-MLV RT activity

Reaction mixtures (25 μl) were comprised of 2 μl of test substance, 0.5 μg of synthetic template-primer poly(rA)*n*-oligo (dT)$_{15}$, 3 μl of 1 mM dTTP, 1 μl of 0.5 μCi [^{3}H]dTTP, and buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 3 μg nuclease-free bovine serum albumin. The reaction was started by adding 5 μl of Mo-MLV RT (2 units) to the reaction mixture. After incubating at 37°C for 30 min, the reaction was terminated by addition of 5 μl of 0.4 M EDTA. 20 μl of reaction mixture was spotted on Whatman DE-81 cellulose paper and washed 6 times with 5% Na_2HPO_4 and 2 times each with water and absolute ethanol. The filters were then air-dried and the radioactivity of the samples was determined with a Beckman 5801 liquid scintillation counter.

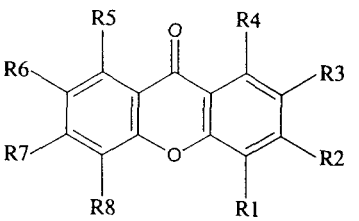
Effects of THXs on DNAP-I activity

30 μl of reaction mixture consisting of 1 μM each of dATP, dGTP and dCTP, 0.5 μCi [^{3}H]dTTP, 1 unit DNAP-I, 10 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 10 μg nuclease-free bovine serum albumin, 1.5 mM dithiothreitol, 5 μg of activated calf thymus DNA and various concentrations of compounds to be tested. The reaction mixture was incubated at 37°C for 30 min, and the samples were then processed for liquid scintillation counting as described (Chu et al., 1992).

Results

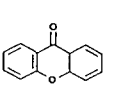
Effects of various THXs on Mo-MLV RT activity

1,3,5,6-THX[1], 1,3,6,7-THX[2], 2,3,6,7-THX[3], 3,4,5,6-THX[4], and 3,4,6,7-THX[5] were examined for anti-Mo-MLV RT activity. In addition,

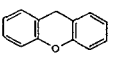


Me: Methyl $-CH_3$
Ac: Acetyl $-COCH_3$
Pr: Propioyl $-COC_2H_5$
Ni: Nicotinyl $-CONC_5H_4$
Be: Benzoyl $-COC_6H_5$
Pa: Palmitoryl $-COC_{15}H_{31}$
Ci: Cinnamoyl $-COCH{=}CHC_6H_5$
Cr: Crotonyl $-COCH{=}CHCH_3$

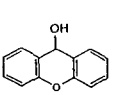
	R1	R2	R3	R4	R5	R6	R7	R8
1,3,5,6-THX	OH	H	OH	H	OH	OH	H	H
2,3,6,7-THX	H	OH	OH	H	H	OH	OH	H
3,4,5,6-THX	H	H	OH	OH	OH	OH	H	H
3,4,6,7-THX	H	H	OH	OH	H	OH	OH	H
Norathyriol(1,3,6,7-THX)	OH	H	OH	H	H	OH	OH	H
1,3,5,6-THX tetraacetate	OAc	H	OAc	H	OAc	OAc	H	H
2,3,6,7-THX tetraacetate	H	OAc	OAc	H	H	OAc	OAc	H
3,4,5,6-THX tetraacetate	H	H	OAc	OAc	OAc	OAc	H	H
3,4,6,7-THX tetraacetate	H	H	OAc	OAc	H	OAC	OAc	H
Norathyriol tetraacetate	OAc	H	OAc	H	H	OAc	OAc	H
Norathyriol tetrapropioate	OPr	H	OPr	H	H	OPr	OPr	H
Norathyriol tetranicotinate	ONi	H	ONi	H	H	ONi	ONi	H
Norathyriol tetrabenzoate	OBe	H	OBe	H	H	OBe	OBe	H
Norathyriol tetrapalmitate	OPa	H	OPa	H	H	OPa	OPa	H
Norathyriol tetracinnamate	OCi	H	OCi	H	H	OCi	OCi	H
Norathyriol tetracrotonate	OCr	H	OCr	H	H	OCr	OCr	H
Norathyriol tetramethoxyxanthone	OMe	H	OMe	H	H	OMe	OMe	H
3,4-dihydroxyxanthone	H	H	OH	OH	H	H	H	H



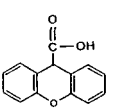
Xanthone



Xanthene



9-hydroxyxanthene



Xanthene-9-carboxylic acid

Fig. 1. The structures of THXs and related compounds.

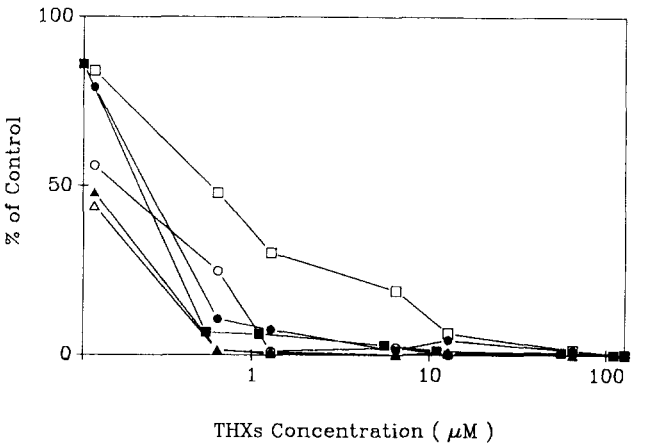


Fig. 2. Inhibition of Mo-MLV reverse transcriptase activity by THXs and quercetin using (rA)*n*-(dT)(15) as template-primer. ○:1,3,5,6-THX; ●:2,3,6,7-THX; △:3,4,5,6-THX; ▲:3,4,6,7-THX; □:Norathyriol; ■:Quercetin.

TABLE 1

Inhibition of Mo-MLV RT activity by THXs, THX derivatives and quercetin using (rA)*n*-$(dT)_{15}$ or (rC)*n*-$(dG)_{12-18}$ as template-primer
a: 1,3,6,7-THX.
–: not determined.

Compounds	IC_{50} (μM)	
	(rA)*n*-$(dT)_{15}$	(rC)*n*-$(dG)_{12-18}$
1,3,5,6-THX	0.154	0.269
2,3,6,7-THX	0.269	0.308
3,4,5,6-THX	0.115	0.154
3,4,6,7-THX	0.115	0.192
Norathyriol[a]	0.577	0.385
1,3,5,6-THX tetraacetate	6.92	–
2,3,6,7-THX tetraacetate	1.36	–
3,4,5,6-THX tetraacetate	1.19	–
3,4,6,7-THX tetraacetate	1.36	–
Norathyriol tetraacetate	2.34	–
Norathyriol tetrapropioate	4.13	–
Norathyriol tetranicotinate	15.53	–
Norathyriol tetrabenzoate	> 50 μM	–
Norathyriol tetrapalmitate	> 50 μM	–
Norathyriol tetracinnamate	> 50 μM	–
Norathyriol tetracrotinate	> 50 μM	–
Norathyriol tetramethoxyxanthone	> 50 μM	–
Xanthone	> 50 μM	–
3,4-dihydroxyxanthone	5.35	–
Xanthene	> 50 μM	–
Xanthene-9-carboxylic acid	> 50 μM	–
9-hydroxyxanthene	> 50 μM	–
Quercetin	0.200	0.298

quercetin, a known RT inhibitor, (Ono et al., 1990; Chu et al., 1992) was also examined. As shown in Fig. 2, the inhibitory activity of compounds [1] to [5] and quercetin on Mo-MLV reverse transcription of a synthetic template-primer, (rA)*n*-$(dT)_{15}$, was concentration-dependent. The IC_{50} values of the 5 THXs were found to range from 0.12 μM to 0.58 μM, while that of quercetin was 0.20 μM (Table 1). When (rC)*n*-$(dG)_{12-18}$ was used as template-primer, the THXs and quercetin were equally potent at inhibiting RT activity as when (rA)*n*-$(dT)_{15}$ was used as template-primer (Table 1).

Effects of acylation of THXs on anti-Mo-MLV RT activity

The effects of acylating THXs on their RT inhibiting activity are shown in Table 1. When the hydroxyl groups of THXs were acetylated, their anti-Mo-MLV RT activity was decreased from 6- to 20-fold. Moreover, when palmitoyl groups were used to block the hydroxyl groups of 1,3,6,7-THX, no anti-RT activity was detected for the derivatives. Aromatic groups, such as benzoyl groups, or cinnamoyl groups also greatly reduced the anti-RT activity of 1,3,6,7-THX. Replacement of the hydroxyl groups of 1,3,6,7-THX with methoxyl groups also eliminated its anti-RT activity (Table 1).

Anti-RT activity of related compounds

Xanthone and 3,4-dihydroxyxanthone were tested for their anti-Mo-MLV RT activity. Xanthone was inactive while 3,4-dihydroxyxanthone was moderately active, with an IC_{50} value of 5.4 μM. Other related compounds such as xanthene, xanthene-9-carboxylic acid and 9-hdroxyxanthene were without anti-RT activity (Table 1).

Mode of inhibition and determination of kinetic constants

The mode of inhibition was studied by changing the concentrations of either the triphosphate substrate or the template-primers. A typical experiment is shown in Fig. 3. All THXs studied were shown to inhibit RT activity by competing with template-primer (rA)*n*-$(dT)_{15}$ but not with the triphosphate substrate, dTTP. K_i values in all cases were determined and are summarized in Table 2.

TABLE 2

K_i values of THXs for Mo-MLV RT with respect to (rA)*n*-$(dT)_{15}$ template-primer and triphosphate substrate, dTTP

THXs	K_i value (μM)	
	(rA)*n*-$(dT)_{15}$	dTTP
1,3,5,6-THX	0.08	0.13
2,3,6,7-THX	0.24	0.43
3,4,5,6-THX	0.06	0.10
3,4,6,7-THX	0.07	0.12

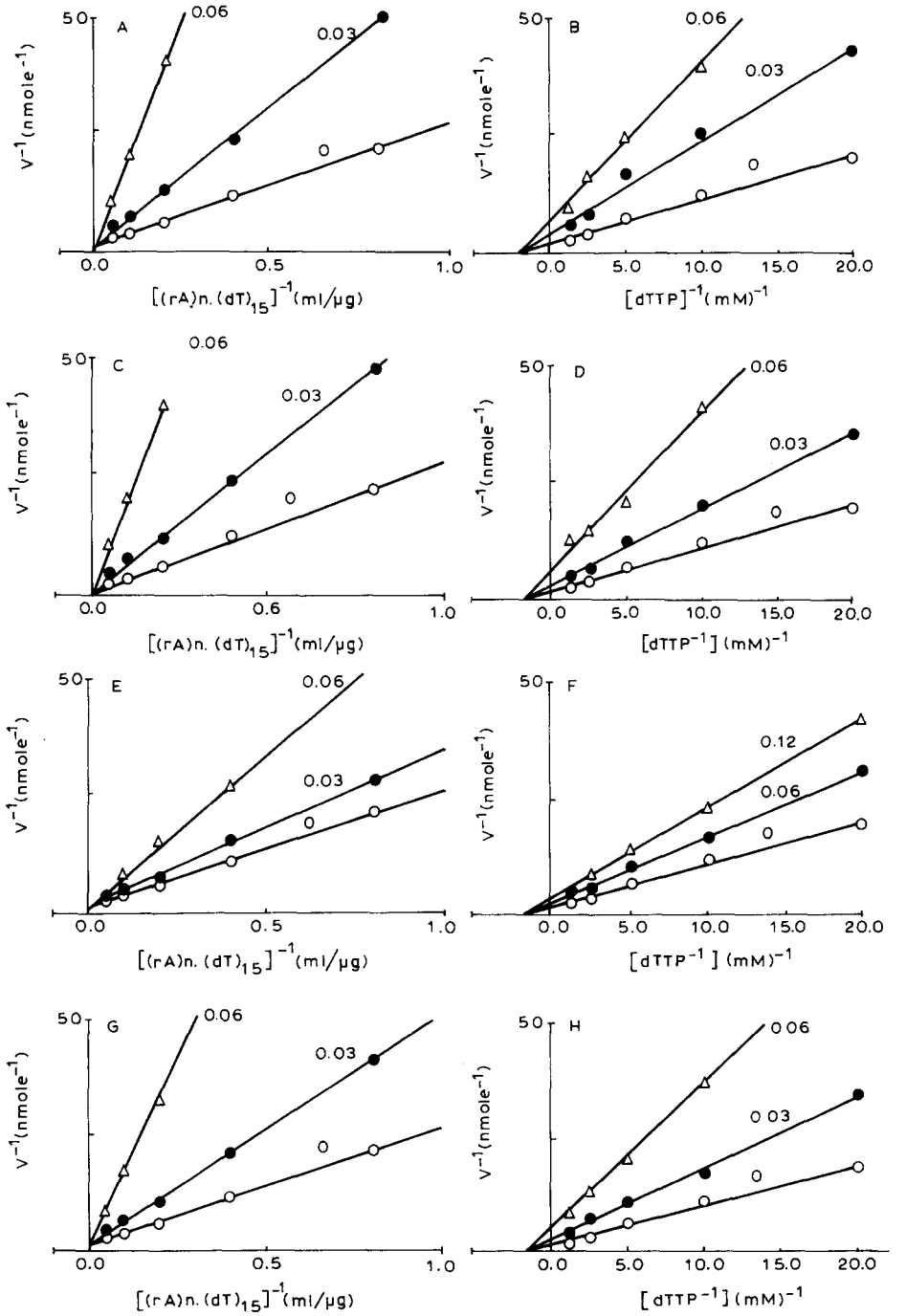

A
B
C
D
E
F
G
H
0.06
0.03
0.12
0
50
0.0
0.5
1.0
5.0
10.0
15.0
20.0
V^{-1}(nmole^{-1})
$[(rA)n.(dT)_{15}]^{-1}$(ml/µg)
$[dTTP]^{-1}$(mM)$^{-1}$

TABLE 3

Effects of THXs on DNAP-I activity
a: 1,3,6,7-THX

THXs	IC_{50} (μM)
1,3,5,6-THX	5.27
2,3,6,7-THX	9.62
3,4,5,6-THX	7.69
3,4,6,7-THX	8.38
Norathyriol[a]	27.88

Effects of THXs on DNAP-I activity

The ability of these five THXs to inhibit DNAP-I activity was also examined. It was found that higher concentrations of THXs were required to inhibit DNAP-I activity than to inhibit Mo-MLV RT activity. As can be seen in Table 3, the IC_{50} values for inhibition of DNAP-I activity were in the range of 5.27 to 9.62 μM, 34 to 72 times higher than required for the inhibition of RT activity.

Discussion

The present experiments demonstrate that THXs, new substances isolated from the Chinese herb *Tripterospermum lanceolatum* (Hyata), possess anti-Mo-MLV RT activity, and that the THXs are as active as naturally occurring flavonoids against RT activity. The five THXs were found to inhibit RT in a concentration-dependent manner with similar IC_{50} values. It is of interest that DNAP-I was about 34- to 72-fold less sensitive to THXs than was Mo-MLV RT, judging from the IC_{50} values of the five THXs for these two enzymes. Lineweaver-Burk plots of Mo-MLV RT activity with regard to various concentrations of dTTP or (rA)*n*-$(dT)_{15}$ template-primer revealed that the five THXs were competitive inhibitors of (rA)*n*-$(dT)_{15}$.

By using several structurally related compounds, some structure–activity relationships were determined for THX inhibition of RT. For example, xanthone, an analogue lacking hydroxyl groups, was unable to inhibit RT activity, while 3,4-dihydroxyxanthone exhibited only weak inhibitory activity with an IC_{50} value of 5.4 μM, 40-fold higher than that of 3,4,5,6-THX. These results suggest that the tetrahydroxyl groups are indispensible for high RT inhibitory activity. Acylation of the four hydroxyl groups of the THXs

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Fig. 3. Analysis of the inhibition of Mo-MLV RT by THXs. Assay conditions are described in Material and Methods. The concentrations (μg/ml) of 3,4,5,6-THX (A,B), 3,4,6,7-THX (C,D), 2,3,6,7-THX (E,F) and 1,3,5,6-THX (G,H) are indicated alongside the plots. The V, [dTTP] and (rA)*n*-(dT)(15) are expressed as nmol [^{3}H]dTMP incorporated per minute, mM and μg/ml, respectively. Specific activity of [^{3}H]dTTP was 16000 dpm/nmol.

considerably decreased their inhibitory activity. For example, the IC_{50} value of 3,4,5,6-THX tetraacetate was 1.19 μM, 17-fold higher than that of 3,4,5,6-THX. Replacement of the hydroxyl groups with larger or more hydrophobic groups, such as palmitoyl or benzoyl groups, totally destroyed the inhibitory activity of the THXs.

The Mo-MLV RT gene has been cloned (Kotewicz et al., 1988) and shown to consist of two separate domains containing polymerase activity (1st to 430th amino acids) and RNase H activity (430th to 550th amino acids). The polymerase domain of human immunodeficiency virus-I (HIV-I) is known to possess an active site to polymerize the growing DNA strand and a binding site with sufficient rigidity to hold the template strand (Lowe et al., 1988; Ferris et al., 1990). It has been proposed that the polymerase domain is a 60–70 Å spheroid with a cylindrical hole or cleft of about 20 Å to accommodate double-stranded nucleic acid (Barber et al., 1990). It is also known that reverse transcriptases are well conserved through evolution. Both HIV-I RT and Mo-MLV RT core region structure, with 21% homology, may be similar (Wain-Hobson et al., 1985; Ruprecht et al., 1986; Johnson et al., 1986). The present investigation shows that THXs are competitive inhibitors with respect to $(rA)_n$-$(dT)_{15}$, suggesting that THXs bind to the site of RT that interacts with double-stranded nucleic acids. Replacement of the tetrahydroxyl groups with bulk structures such as palmitoyl or benzoyl groups may have caused steric hindrance, resulting in the observed decrease of anti-RT activity of these derivatives. It has also been suggested that two lysine and aspartic acid residues reside in the conserved segment of the polymerase domain, and that these two segments form α-helices at the double-strand nucleic acid binding site (Barber et al., 1990). Our results, showing that the tetrahydroxyl groups of THXs are essential for their strong inhibitory activity against RT, suggest that these hydroxyl groups may be involved in the interaction between the conserved segment of the polymerase portion of RT and THXs.

Acknowledgements

Present investigation was supported in part by Grant NSC-80-0420-B002-27 from the National Science Council, Republic of China. We would also like to express our appreciation to Dr. Steve R. Roffler for his critical and valuable suggestions regarding preparation of this paper.

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