

Differential *in vitro* Anti-HIV Activity of Natural Lignans

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Two naturally occurring lignanoides, isolated from the tropical climbing shrub *Ipomoea cairica*, (–)-arctigenin and (–)-trachelogenin, were found to inhibit strongly replication of human immunodeficiency virus type 1 (HIV-1; strain HTLV-III B) *in vitro*. At a concentration of 0.5 μM , (–)-arctigenin and (–)-trachelogenin inhibited the expression of HIV-1 proteins p17 and p24 by 80–90% and 60–70%, respectively. The reverse transcriptase activity in the culture fluids was reduced by 80–90% when the cells (HTLV-III B/H9) were cultivated in the presence of 0.5 μM (–)-arctigenin or 1 μM (–)-trachelogenin. At the same concentrations, the formation of syncytia in the HTLV-III B/H9-Jurkat cell system was inhibited by the compounds by more than 80%. A series of other lignan type compounds displayed no anti-HIV activity. Studying the molecular mechanism of action of (–)-arctigenin and (–)-trachelogenin we found that both compounds are efficient inhibitors of the nuclear matrix-associated DNA topoisomerase II activity, particularly of the enzyme from HIV-1-infected cells. Our results suggest that both compounds prevent the increase of topoisomerase II activity, involved in virus replication, after infection of cells with HIV-1.

Introduction

Lignans are naturally occurring secondary metabolites formed by enzymatic dimerization of phenylpropanoid precursors (mostly cinnamyl alcohols such as coniferyl alcohol). The first linkage is formed between the side chains C-2 of both monomers. By introduction of further linkages several types of lignans may be produced (Fig. 1). The biological significance of these compounds is not yet fully clear. For instance wounded conifers excrete balsams containing lignans. Certain lignans inhibit the germination of seeds or are powerful irritants (e.g. podophyllotoxin) repelling potential predators [1]. Dibenzylbutanolide type lignans conjugated to glucuronic acid were also identified as urinary constituents in man; a relationship of these lignans and the function of gonads/ovaries has been proposed [2].

Certain natural cyclolignanoid type compounds, such as podophyllotoxin, act as spindle poisons inhibiting microtubule assembly [3]. On the other hand, the semisynthetic podophyllotoxin derivative, etoposide, which is clinically used as a cytostatic agent [4], probably possesses another mechanism of action through which it inhibits cell proliferation: it has been shown to interfere with the activity of topoisomerase II [5]. *E.g.* the inhibitory effect of etoposide on plaque formation by *Herpes simplex* virus in cultured cells has been attributed to an impairment of function of host cell topoisomerase II [6]. However, in contrast to podophyllotoxin which displays a considerable *in vitro* anti-*Herpes simplex* virus effect, etoposide was active only at very high concentrations [7].

In a tropical climbing shrub, *Ipomoea cairica* (L.) SWEET (Convolvulaceae), we discovered two lignanoides [8] which we could isolate from the above-ground parts and characterize as (–)-arctigenin [9] and (–)-trachelogenin [10]. Here we report that these 2,3-dibenzylbutanolide type com-

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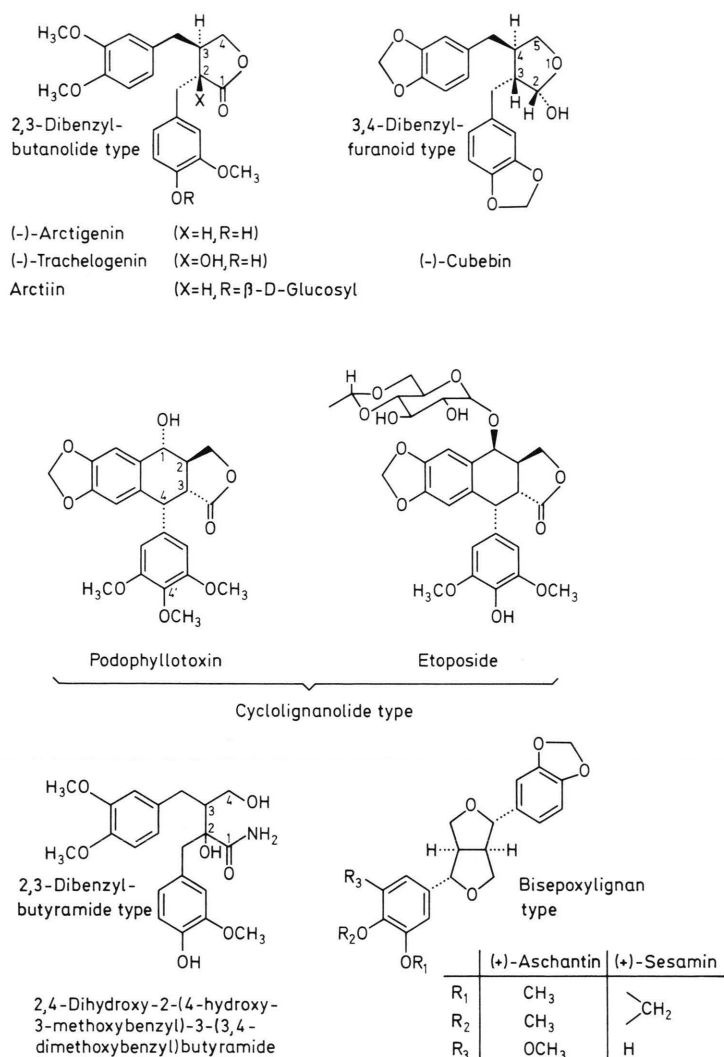


Fig. 1. Classification and chemical structure of the lignans cited in the text. 2,3-Dibenzylbutanolide type: (-)-arctigenin, (-)-trachelogenin, and arctiin; 3,4-dibenzylfuranoid type: (-)-cubebin; 2,3-dibenzylbutyramide type (not natural): 2,4-dihydroxy-2-(4-hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)butyramide; cyclolignanoid type: podophyllotoxin; and bisepoxy lignan type: (+)-aschantin and (+)-sesamin. The numbering depends on the specific cyclic system and is therefore not comparable between the different types.

pounds exhibit a strong anti-HIV activity *in vitro*, while a group of different types of lignans which has been included in these studies were without effect. Previously we determined that infection of H9 cells with HIV-1 *in vitro* results in a transient decrease of DNA topoisomerase II activity (at day 1–3), followed by a drastic increase at day 4 [11]. Simultaneously, the production of HIV-1 strongly increased. The topoisomerase II represents a major component of the nuclear matrix [12, 13]. Our studies presented in this report revealed that (-)-arctigenin and (-)-trachelogenin efficiently inhibit both nuclear and nuclear matrix-associated

topoisomerase II activity from uninfected and HIV-infected cells.

Materials and Methods

Compounds

(-)-Arctigenin (mol. wt. 372.1) and (-)-trachelogenin (mol. wt. 388.2) were isolated from the above-ground parts of *Ipomoea cairica* (L.) SWEET; arctiin (mol. wt. 534.3) from the fruits of *Arctium lappa* L. [14]; (-)-cubebin (mol. wt. 356.4) from the fruits of *Piper cubeba* L. f.; aschantin (mol. wt. 370.4) and (+)-sesamin (mol. wt. 354.3)

from the fruits of *Piper guineense* SCHUMACH. *et* THONN. [15]. 2,4-Dihydroxy-2-(4-hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)butyramide (mol. wt. 405.4) was obtained from (–)-trachelogenin by treatment with ammonia (to be published). Podophyllotoxin (mol. wt. 414.4) was obtained from Roth, Karlsruhe (F.R.G.). The structural formulae of these compounds are shown in Fig. 1.

Plasmid pBR322 DNA was isolated from the bacteria by lysis with SDS and by CsCl equilibrium gradient sedimentation in the presence of ethidium bromide, as described [16].

Cell growth inhibition studies

L5178y mouse lymphoma cells were grown in Eagle's minimum essential medium, supplemented with 10% horse serum in roller tube cultures [17, 18]. Dose response experiments were performed as described [18]. The cell concentration was determined electronically (Cytocomp Counter, model Michaelis). The ED₅₀ (inhibition concentration, causing 50% inhibition of cell growth) was estimated by logit regression [19].

HTLV-III B and H9 cells

The culture conditions of the uninfected and HIV-1 (strain HTLV-III B)-infected H9 cells (human T-cell line, clone 9) [20] and of the Jurkat cell system [21] were as described. H9 cells were maintained in RPMI 1640 medium containing 20% fetal calf serum. H9 cells (5×10^5 cells/ml) were infected with 1.6×10^6 HTLV-III B particles, obtained from culture supernatants of virus producing H9 cells [22]. Cultivation was performed for 4 days in the absence or presence of the compounds. The compounds were dissolved in dimethylsulfoxide at a stock concentration of 2 mM; they were added 5 h after addition of the virus (= day 0) to the uninfected or infected cultures. The final concentration of dimethylsulfoxide was less than 0.1%.

The percentage of cells expressing HIV-1 p17 and p24 *gag* protein was determined by indirect immunofluorescence microscopy [23]; under the conditions used the reactivity of the monoclonal antibodies used with untreated HIV-1-infected H9 cells was 37% (anti-p17) and 44% (anti-p24), respectively. As a measure for virus production, the

reverse transcriptase activity was determined in the culture supernatants, as described [23, 24]. In the supernatant from untreated cells, infected for 4 days with HIV-1, the activity of this enzyme was 5.7×10^5 cpm/ml culture fluid. This value and the number of p17- and p24-positive cells in the untreated HIV-1-infected control cultures were set at 100%.

The syncytium-induction assay was performed as described [25]. HTLV-III B-infected H9 cells (1×10^5 cells) incubated for 4 days in the absence or presence of compound were mixed with 1×10^5 uninfected Jurkat cells in a final volume of 100 μ l.

Preparation of nuclei and nuclear matrix

Nuclei were isolated from uninfected and HIV-1-infected H9 cells by the method of Blobel and Potter [26], except that 1 mM phenylmethanesulfonyl fluoride and 5 mM 2-mercaptoethanol were added to all the buffers used. Nuclear matrices were prepared as described previously [27, 28]; 0.12 mg of matrix protein was determined to correspond to 10^6 nuclei. The nuclei and nuclear matrices were inspected electron microscopically; no differences between these substructures from uninfected and HIV-1-infected cells were observed [29, 30].

Nuclear extracts for determination of nuclear DNA topoisomerase II activity were prepared by treatment of nuclei with 30 mM Tris-HCl (pH 7.9), 350 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM sodium metabisulfite (pH 7.0), 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, as described [31, 32].

DNA topoisomerase II assay

DNA topoisomerase II activity was determined by measuring the relaxation of supercoiled plasmid pBR322 DNA in the presence of ATP, essentially as described [33]. The standard reaction mixture contained in a final volume of 40 μ l, 0.6 μ g of negatively supercoiled circular pBR322 DNA (containing <10% nicked circles) and 1 mM ATP in 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 50 mM KCl, 50 mM NaCl, 15 μ g/ml of bovine serum albumin. Either nuclear extract (5 μ l) from 7×10^4 nuclei or 8 μ g (with respect to protein) of nuclear matrix preparation were added to the standard assay. After incubation for 0–30 min at 30 °C in the absence or presence of compound, reactions were ter-

minated as described previously [13]. The reaction products were analyzed by electrophoresis in 1% agarose gels followed by ethidium bromide staining. DNA bands visible under UV light were photographed using a Polaroid 665 film. The percentage of DNA relaxed was quantitated by scanning densitometry as previously described [13, 32, 33].

Protein was determined as described by Lowry *et al.* [34] using bovine serum albumin as a standard.

Results and Discussion

As shown in Table I, the 2,3-dibenzylbutanolides (–)-arctigenin and its natural 2-hydroxy derivative (–)-trachelogenin are potent anti-HIV agents *in vitro*. Using the H9/HTLV-III B system, (–)-arctigenin at the low concentration of 0.1 µg/ml (0.27 µM) was found to reduce the expression of viral p17 and p24 proteins by 60–70%; (–)-trachelogenin at 0.2 µg/ml (0.52 µM) inhibited the expression of these proteins by 60–70% (Table I). At the same concentrations, the reverse transcriptase activity in the culture supernatants (as a measure of production of HIV-1) was reduced to 69% and 57%, respectively. At higher concentrations (>1–2 µM), the compounds are toxic for the cells. (–)-Arctigenin and (–)-trachelogenin efficiently abolished cell fusion with Jurkat cells (by 85%) at a concentration of 0.2 µg/ml (0.54 µM) and 0.3 µg/ml (0.77 µM), respectively.

A series of other types of lignans, most of them closely related to arctigenin and trachelogenin, do not exhibit any anti-HIV activity (Table I). Even arctiin, the β-D-glucoside of (–)-arctigenin, was inactive. From these results we conclude that only very small variations in the structure of the molecule lead to a complete loss of anti-HIV activity. As arctiin was inactive, a free phenolic hydroxyl at C-4 of the substituted benzyl moiety at C-2 might be important for the anti-HIV activity of (–)-arctigenin and (–)-trachelogenin. The lack of activity of the butyramide derivative indicates that a lactone ring might be necessary, particularly because (–)-cubebin, which can be considered as a simple reduction product of the corresponding lactone, was also inactive. Finally, it is remarkable that the cyclolignan type compound podophyllotoxin displays no activity. From this fact we assume that the additional ring closure between the side chain

Table I. Anti-HIV-1 activity of different lignan type compounds in the HTLV-III B/H9 cell system *in vitro*. H9 cells were infected with HTLV-III B for 4 days. The effect of the compounds on HIV-1-induced Jurkat cell fusion, the expression of HIV-1 p17 and p24 protein and the reverse transcriptase (RT) activity in the supernatants were measured at day 4 as described in Materials and Methods. The number of syncytia as well as the expression of viral p17 and p24 and the RT activity in untreated HIV-1-infected control cultures was set at 100%. The means of 5 parallel experiments are given. DHDB: 2,4-dihydroxy-2-(4-hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)-butyramide.

Compound	Conc. [µM]	Percentage of			
		Syncytia	p17	p24	RT
None	–	100	100	100	100
(–)-Arctigenin	0.13	81	95	95	69
	0.27	25	34	42	26
	0.54	15	17	12	11
	1.07 (toxic)	24	17	16	58
(–)-Trachelogenin	0.52	23	31	40	57
	0.77	15	12	12	31
	1.03	15	10	10	18
	2.06 (toxic)	23	20	22	55
Arctiin	5.0	93	88	96	86
	10.9	87	93	90	83
(–)-Cubebin	12.7	86	96	91	97
	31.6	96	84	87	92
DHDB	1.8	93	87	84	89
	5.9 (toxic)	83	94	97	91
Podophyllotoxin	0.007	84	86	89	74
	0.03 (toxic)	81	87	92	97
(+)–Aschantin	10.6	97	82	85	71
	31.3 (toxic)	85	93	86	83
(+)–Sesamin	8.2	84	93	97	81
	38.5 (toxic)	89	91	82	73

of the second phenylpropanoid monomer and the aromatic moiety of the first monomer is accompanied by a complete loss of activity. However, additional studies including further derivatives with different combinations of structural variations will be necessary to corroborate these assumptions.

The results from the cell growth inhibition studies of the different lignan type compounds in the L 5178y mouse lymphoma cell system are summarized in Table II. Our results indicate that the cytostatic activity and anti-HIV activity of the lignans are based on different mechanisms of action: podophyllotoxin is a strong cytostatic agent (Table II), while it was found to be inactive against

Table II. Cytostatic activity of different lignan type compounds in L 5178 y mouse lymphoma cells *in vitro*.

Compound	ED ₅₀ conc. [μM]
(-)-Arctigenin	1.16
(-)-Trachelogenin	2.01
Arctiin	≥20
(-)-Cubebin	63.2
2,4-Dihydroxy-2-(4-hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)-butyramide	3.45
Podophyllotoxin	0.007
(+)-Aschantin	47.3
(+)-Sesamin	42.4

HIV (Table I). Furthermore, (-)-arctigenin, (-)-trachelogenin and its 2,3-dibenzylbutyramide derivative showed remarkable and comparable cytostatic properties (Table II); however, only the first two compounds but not the butyramide displayed anti-HIV activity (Table I).

Previously we determined that during the initial phase of infection (at day 1–3) of H9 cells with HIV-1 *in vitro* nuclear DNA topoisomerase II activity decreased and then, at day 4, strongly increased [11]. The increase in topoisomerase II activity coincided with the onset of virus production [11]. The underlying mechanism of these alterations in DNA topoisomerase II activity was found most likely to be a change in the phosphorylation state of the enzyme [11]. DNA topoisomerase II has been shown to be a phosphoprotein [31, 32, 35–37]. It is phosphorylated *in vitro* by casein kinase II [35], nuclear protein kinase N II [32] or protein kinase C [32, 36, 37]. Phosphorylation of the enzyme, resulting in an enhanced enzyme activity, has also been demonstrated *in vivo* [31]. The phosphorylation state of the enzyme has been shown to be correlated with its DNA relaxing activity [31, 32, 35–37]. The lower level of topoisomerase II activity in the early phase of infection may be due to an impairment of the phosphatidylinositol/protein kinase C pathway, caused by components of HIV-1 [38], resulting in a reduced phosphorylation of nuclear matrix-associated DNA topoisomerase II and hence in an inhibition of this enzyme [39]. Inhibition of protein kinase C-mediated phosphorylation of DNA topoisomerase II has been demonstrated to result in an inhibition of HIV-1 production [11].

In the following experiments, DNA topoisomerase II activity was determined in the nuclear matrix of H9 cells, which contains a large portion of the type II enzyme activity, present in the nucleus, but only a small amount of nuclear topoisomerase I activity [12, 13, 32]. The measurements were performed using H9 cells infected for 4 days by HIV-1, which contain a high level of DNA topoisomerase II activity [19] as well as in uninfected cells. (-)-Arctigenin and (-)-trachelogenin were found to cause a strong inhibition of the matrix-associated enzyme activity (Fig. 2). The conversion rate of the bacterial plasmid DNA after an incubation period of 10 min from the fully supercoiled circular form into the relaxed forms, measured with matrix preparation from uninfected cells, was 63% (Fig. 2A) and that with matrix from infected cells, 81% (Fig. 2B). In the presence of 1 μM (-)-arctigenin or 1 μM (-)-trachelogenin the DNA-relaxing activity of the enzyme from un-

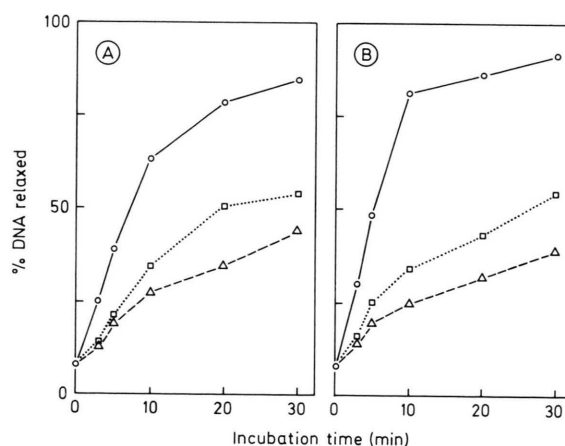


Fig. 2. Effect of (-)-arctigenin and (-)-trachelogenin on time course of relaxation of negatively supercoiled pBR 322 DNA from uninfected and HIV-1-infected H9 cells. Nuclear matrices were prepared from uninfected (A) and HIV-1-infected H9 cells (B) after an incubation period of 4 days. The matrices (8 μg of protein/assay) were preincubated for 10 min at 4 °C in reaction mixture without ATP in the absence (O) or presence of 1 μM (-)-arctigenin (Δ) or 1 μM (-)-trachelogenin (□). Reactions were started by addition of ATP and incubation was performed at 30 °C for up to 30 min. Each assay contained 0.6 μg of pBR 322 DNA. The relaxation of the plasmid DNA was quantitated by scanning densitometry. The values are given in percent DNA relaxed and present means of 3 independent experiments; the S.D. was less than 8%.

Table III. Effect of different lignan type compounds on DNA topoisomerase II activity present in nuclear extracts and nuclear matrix from HIV-1-infected H9 cells. The determination of enzyme activity was performed after an incubation period of 10 min. Subcellular fractions of HIV-1-infected H9 cells at day 4 were used. The activity of the enzyme determined in the absence of compound was arbitrarily set at 1.0. For further details, see legend to Fig. 2. The means of 3 independent experiments, each done in triplicate, are given; the S.D. were less than 10%.

Compound	Conc. [μM]	DNA topoisomerase II activity (arbitrary units)	
		Nuclear extract	Nuclear matrix
None	—	1.00	1.00
(–)-Arctigenin	0.1	0.94	0.90
	0.3	0.63	0.51
	1.0	0.30	0.23
(–)-Trachelogenin	0.1	1.01	0.97
	0.3	0.91	0.85
	1.0	0.40	0.36

infected cells was reduced to 35% and 49%, respectively (calculated after subtraction of the percentage amount of used plasmid being not present in the fully supercoiled circular form). Interestingly, a significantly stronger inhibition was found for topoisomerase II from HIV-infected cells; at the same concentrations the activity of the matrix-associated enzyme was decreased by the compounds to 23% and 36%, respectively. An inhibition of topoisomerase II by (–)-arctigenin and (–)-trachelogenin was found not only for the matrix-associated enzyme, but also for the enzyme in total nuclear extracts (Table III). (–)-Trachelogenin was less efficient than (–)-arctigenin. Even at a concentration of 10 μM, none of the other tested lignan type compounds displayed an inhibitory effect on topoisomerase II (Table III).

From our results showing that the activity of topoisomerase II correlates with HIV-1 production [11], inhibition of topoisomerase II by com-

pounds, such as (–)-arctigenin or (–)-trachelogenin, should be a promising strategy in treatment of HIV infection. Future studies must show whether the stronger inhibition of DNA-relaxing activity found in HIV-infected cells is due to a modification of topoisomerase II or expression of a novel species of topoisomerase, as indicated in some studies [40]. The fact that the cytostatic activity and anti-HIV activity of lignans are apparently caused by different mechanisms of action of the compounds offers the possibility to develop analogs possessing a strong anti-HIV activity and a comparatively low cytotoxicity.

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