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TOPOISOMERASE II: A POTENTIAL TARGET FOR NOVEL ANTIFUNGAL AGENTS

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Summary: Several podophyllotoxin-related lignans have been shown to possess significant antifungal activity against a number of filamentous fungi. Initial structure-activity studies indicate this action is sensitive to change at the 4 and 4' positions of the podophyllotoxin skeleton. Good correlation has been observed between antifungal action and the ability to inhibit the relaxation of supercoiled plasmid DNA by a topoisomerase II preparation from *Saccharomyces cerevisiae*. Etoposide, an inhibitor of mammalian topoisomerase II, is inactive against this yeast enzyme, although good inhibition is shown by amiloride, 4'-(9-acridinylamino)-methanesulphon-*m*-anisidide (*m*-AMSA) and novobiocin, known inhibitors of the mammalian enzyme.

The topology of both prokaryotic and eukaryotic cellular DNA is under the control of a family of enzymes, the so called topoisomerases or bacterial gyrases (1,2). The potential of these enzyme systems to serve as targets for chemotherapeutic agents is increasingly being recognised following the realisation that several important medicinal agents exert their action, at least in part, by inhibition of the type II topoisomerase enzyme system (3). In the prokaryotes, these gyrase inhibitors include the coumarin antibiotics such as novobiocin and the quinolone antibiotics, e.g. nalidixic acid, whilst in the eukaryotes they number several cytotoxic agents, including the glycosidic lignan derivative etoposide (VP 16-213). This latter compound, a semi-synthetic derivative from the naturally occurring cytotoxic lignan podophyllotoxin, shows markedly greater inhibition of mammalian topoisomerase II than the parent compound. Indeed, podophyllotoxin is thought to exert its cytotoxicity through a process of tubulin binding rather than topoisomerase inhibition (4). Previous structure-activity studies involving podophyllotoxin derivatives (5) have indicated that a 4'-OH group together with a 4 β-substituted ether give maximal *in vitro* action against the mammalian topoisomerase II enzyme.

This communication reports initial structure-activity studies on the previously unreported antifungal action of podophyllotoxin derivatives and, using topoisomerase II isolated from *Saccharomyces cerevisiae*, considers the possible correlation of this antifungal activity with an

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ability to inhibit this enzyme *in vitro*. The sensitivity of the *Saccharomyces* enzyme system to selected inhibitors of mammalian topoisomerase II is also reported.

MATERIALS AND METHODS

Compounds: Podophyllotoxin was isolated from *Podophyllum hexandrum* according to the procedures reported previously (6). Semi-synthetic derivatives (1-3, 5-8) were prepared from podophyllotoxin (4) as described previously (6,7). Nalidixic acid, novobiocin, amiloride and griseofulvin were purchased from the Sigma Chemical Co. Etoposide (9) was supplied by the Bristol-Myers Co.

Topoisomerase II: *Saccharcmyces cerevisiae* was grown in Malt Extract Broth at 25°C for 2 days, harvested by centrifugation, resuspended in Tris-EDTA-glycerol (TEG) buffer and disrupted by the method of Figgitt *et al* (8). Topoisomerase II was isolated and purified by a previously reported (8) modification of the method of Goto *et al* (9).

Assay of Enzyme Activity: Topoisomerase II enzyme activity was assayed by monitoring the relaxation of supercoiled plasmid DNA (pBR325), according to the following modification of a procedure reported by Goto *et al* (9). Enzyme reactions were performed in a total reaction volume of 20 µl. Compounds under test were freshly prepared in 10% DMSO (v/v) in HEPES buffer (pH 6.7, 0.1M), with a corresponding volume (5 µl) of 10% DMSO (v/v) in HEPES buffer being used for the controls. The reaction was quenched by addition of 3 µl of a solution of SDS (1%, w/v), bromophenol blue (0.5%, w/v) and glycerol (87%, v/v). The extent of relaxation was followed by submarine agarose gel (0.7% w/v) electrophoresis (Bio-Rad Mini Sub DNA Cell) performed at 60 V for 2 hr and the bands visualised under UV light (302 nm) after staining with ethidium bromide (10 µg/ml) for 10 mins. Photographs were recorded with an MP-3 polaroid camera. Sufficient enzyme was included in the assays to ensure complete relaxation in control experiments within a 30-45 min period [relaxation time (RT) = 45 min.]. Enzyme inhibition was thus indicated by an increase in the relaxation time following addition of the test compound. Assays were performed at 0, 30, 45, 60, 90, 120 and 180 min. of drug contact and the RT recorded as the time at which complete relaxation was first observed.

Plasmid DNA pBR325: This plasmid was isolated from *E. coli* HB101 by the caesium chloride/ethidium bromide ultracentrifugation procedure of Clevell and Helinski (10).

Antifungal Activity: Antifungal activity was assessed by zone diffusion in seeded Potato Dextrose Agar employing 100 µl of drug solution in 8 mm diameter wells. Compounds under test were dissolved in DMSO (50%, v/v in distilled water). Where activity was seen, an estimate of the MIC (minimum inhibitory concentration) was made by extrapolation from the logarithmic relationship between concentration and zone of inhibition.

RESULTS AND DISCUSSION

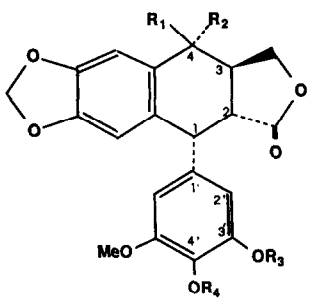
Podophyllotoxin and related lignan derivatives exhibit a wide range of biological effects including antiviral, ichthyotoxic, plant-growth inhibitory, insecticidal and antitumour activity. The data in Table 1, however, represent the first report of significant antifungal activity within this group of compounds. Preliminary experiments with semi-synthetic 4'-demethyldesoxypodophyllotoxin (1) have indicated activity against other filamentous fungi including *Calcarisporium thermophilum, Epidermophyton floccosum, Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes*, as well as those listed in Table 1. Of those organisms found to be most sensitive, *Geotrichum flavo-brunneum* was chosen as the most suitable challenge organism for initial structure-activity studies on the podophyllotoxin derivatives. In these tests, 4'-demethylpodophyllotoxone (2) and 3',4'-didemethyldesoxypodophyllotoxin (3) were the only other compounds to display significant

Table 1. Antifungal activity of podophyllotoxin derivatives (1-3)

Organism	MIC (μg/ml)		
	(1)	(2)	(3)
Aspergillus niger	80	40	80
Geotrichum flavo-brunneum	3	3	100
Microsporum canis	12	ND	ND
Fusarium culmorum	12	ND	ND
Trichophyton erinacei	1	10	20

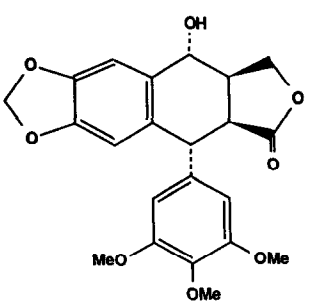
ND= not determined

antifungal activity. All the other compounds evaluated, including podophyllotoxin (4), 4'-demethylpodophyllotoxin (5), desoxypodophyllotoxin (6), podophyllotoxone (7), picropodophyllin (8), and etoposide (9) were considered essentially inactive since no inhibitory effects were observed below concentrations of 1000 μg/ml. Clearly, these data indicate that within the podophyllotoxin-related group, a number of structural features appear necessary for maximal antifungal inhibitory action. In particular, the activity appears sensitive to changes at positions 4 and 4', in a manner analogous to structure-activity profiles seen in the inhibition of mammalian topoisomerase II (5). Consequently, a potential target for these compounds was considered to be fungal topoisomerase II (Compounds 1-9).

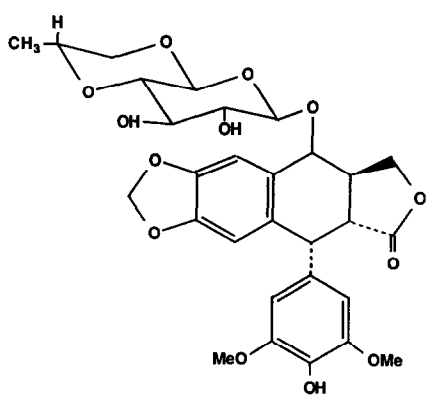


Compound	R_1	R_2	R_3	R_4
(1)	H	H	Me	H
(2)		=O	Me	H
(3)	H	H	H	H
(4)	H	OH	Me	Me
(5)	H	OH	Me	H
(6)	H	H	Me	Me
(7)		=O	Me	Me

(1~7)



(8)



(9)

Compounds 1-9

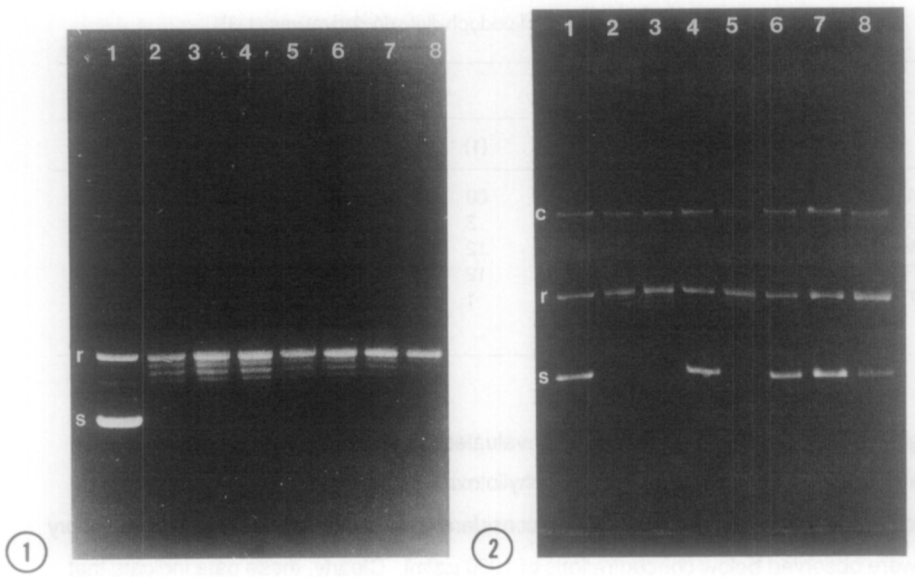


Fig. 1. Time dependency assay of *S. cerevisiae* DNA topoisomerase II.
Lane (1) = control (pBR325). Lanes (2) - (8) = incubation of pBR325 with enzyme preparation, reaction terminated at 15, 30, 45, 60, 90, 120 and 180 min. respectively.
s = supercoiled DNA, r = relaxed DNA.

Fig. 2 . Effects of selected compounds on *S. cerevisiae* DNA topoisomerase II activity.
Lane [1] = control (pBR325). Lane [2] = pBR325 incubated with enzyme preparation.
Lanes [3] - [8] = incubation of pBR325 with enzyme preparation
in the presence of : [3] etoposide (**9**), [4] amiloride, [5] podophyllotoxin (**4**),
[6] 3',4'-didemethyldesoxypodophyllotoxin (**3**),
[7] 4'-demethyldesoxypodophyllotoxin (**1**) and [8] 4'-demethylpodophyllotoxone (**2**).
Incubation time = 180 min. Compound concentration = 10 µg/ml.
s = supercoiled DNA, r = relaxed DNA, c = chromosomal DNA.

Although at the present time the precise cellular functions of topoisomerase enzyme systems are poorly understood, there are several *in vitro* enzyme assay procedures which can be used to demonstrate their ability to induce topological changes in DNA structure. The relaxation of supercoiled plasmid DNA by an ATP-dependent topoisomerase II from *Saccharomyces cerevisiae* was chosen as the enzyme reaction against which to evaluate these compounds (Figs 1 and 2). The data presented in Table 2 show a similar trend to the order of antifungal activity, in that the essential requirements for successful inhibition of topoisomerase II focus around changes at positions 4 and 4', with a 4'-OH group mandatory for good inhibitory action. Most important is the observation that the podophyllotoxin derivatives (**1**), (**2**) and (**3**), identified as antifungal agents, are the most potent topoisomerase inhibitors in the series. Somewhat surprisingly, etoposide, generally regarded as an inhibitor of mammalian topoisomerase II, showed no activity in this assay. Whether this reflects differential sensitivities in these topoisomerases or whether metabolic activation is necessary for the inhibitory action of etoposide is currently under investigation.

Table 2. Inhibition of topoisomerase II from *Saccharomyces cerevisiae* by selected compounds at the given concentrations (Control RT = 45 min)

Compound	Relaxation Time (RT) (Min.) 10μg/ml	100ng/ml
(1)	>180	120
(2)	>180	60
(3)	>180	90
(4)	120	45
(5)	60	45
(6)	60	45
(7)	60	45
(8)	45	45
Etoposide (9)	45	45
Nalidixic Acid	45	45
Amiloride	>180	>180
Novobiocin	>180	45
Griseofulvin	45	45
m-AMSA	>180	120

In addition to the podophyllotoxin analogues, several other agents have been evaluated in this fungal topoisomerase system (Table 2). Neither nalidixic acid, a bacterial gyrase inhibitor, nor griseofulvin, an antifungal tubulin-binding agent, were active, but the two inhibitors of mammalian topoisomerase II, novobiocin and *m*-AMSA, did show inhibitory effects. Perhaps most surprising was the potency of amiloride as a topoisomerase II inhibitor. A previous report (11) has shown this pyrazine diuretic to be an inhibitor of mammalian topoisomerase II, albeit significantly weaker than the other recognised inhibitors such as etoposide and *m*-AMSA, as measured by their ability to inhibit the enzyme-mediated relaxation of supercoiled plasmid DNA. A recent communication has also shown amiloride to be active against bacterial gyrase (12). Against topoisomerase II from *Saccharomyces cerevisiae* however, it was the most potent compound evaluated by us, although we have not been able to demonstrate any intrinsic antifungal activity. The strongly basic nature of the acylguanidino side-chain may result in reduced cell penetration which would account for this. The use of *Saccharomyces* mutants with increased cell wall permeability has recently been described as a fungal model in which the actions of both topoisomerase type I and II inhibitors may be studied (13).

Although yeasts have been suggested as suitable systems for studying the function of eukaryotic DNA topoisomerases, clearly differences do exist between yeast and mammalian topoisomerase II, as determined by their sensitivities to various inhibitors, a fact which should not be overlooked when extrapolating from one system to the other. Furthermore, the correlation of antifungal activity with topoisomerase II inhibition in a series of podophyllotoxin derivative implicates this enzyme system as a possible primary target for these compounds. We believe that the demonstration of differential sensitivities between the mammalian and fungal enzyme systems, coupled with the potential for such inhibitors to cause cell death, offers the medicinal chemist an important new target for the development of novel antifungal agents.

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