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Short communication

The cytotoxicity of T-2 toxin and related 12,13-epoxytrichothecenes to Adriamycin-sensitive and -resistant P388 leukemia cells

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Summary. The cytotoxicity of T-2 toxin and related trichothecenes was studied in Adriamycin-sensitive and -resistant P388 leukemia cells in vitro. The structure-activity relationship indicated that a free hydroxyl in the C-3 position contributed to the activity. Free hydroxyls at the 4, 8, and 15 positions interfered with the activity, and their estrification resulted in improved cytotoxicity. The cytotoxic activity of these trichothecenes did not seem to be related to their degree of lipophilicity. Adriamycin-resistant P388 cells were cross-resistant to the trichothecenes, and this resistance could be circumvented by verapamil.

Introduction

The trichothecenes are sesquiterpenoid mycotoxins characterized by the 12,13-epoxy-trichothec-9-ene ring system. T-2 toxin, the most widely known member of the trichothecenes, has shown a wide range of biological activities, including a significant antitumor activity against P388 murine leukemia [1, 3, 7, 13]. The trichothecenes have been divided into two classes: (a) simple type (e.g., T-2 toxin), and (b) compounds that contain a macrocyclic diester bridge between carbons 4 and 5 (e.g., verrucarin A, roridin A). A subgroup of the simple trichothecenes are derivatives that have a carbonyl at carbon 8; the cytotoxicity of this subgroup against HeLa tumor cells has been reported to be lower than that of the other simple trichothecenes [15].

Grove and Mortimer [5] have found that the reductive opening of the 12,13-epoxy group of 3,4,15-triacethylscirpenol resulted in a 200-fold reduction in the cytotoxic activity against HEp2, a malignant cell line, indicating the importance of the 12,13-epoxide to the cytotoxic activity. However, in their study the role of the substituents in positions 3, 4, 8, and 15 to the cytotoxic activity could not be determined.

A considerable number of trichothecenes have been screened for antitumor activity in mice inoculated with P388 murine leukemia cells [1, 3]. It was pointed out that the evaluation of the data obtained for the analysis of the structure-activity relationship had to take into account not only the antitumor activity but also the involvement of other factors such as the therapeutic ratio and potency of the trichothecenes [4]. Furthermore, the trichothecenes have ester groups that may undergo rapid in vivo cleavage by the liver esterases [4], which might further complicate the evaluation of the cytotoxic activity of the trichothecenes against tumor cells in vivo. Therefore, it seemed that a study of the growth inhibitory activity of the trichothecenes against tumor cells in vitro would allow a more direct analysis of the structure-activity relationship. The major emphasis in the present study was on finding out whether the ester groups or the free hydroxyls of the trichothecenes contributed to or interfered with the growth inhibitory activity.

The increase in life span following the treatment of mice bearing Adriamycin-resistant P388 cells (P388/ADR) with T-2 toxin or 4,15-diacetylscirpenol has been reported to be much smaller than that observed in mice inoculated with Adriamycin-sensitive P388 cells [2, 7, 13]; however, no attempt was made to quantify the degree of this cross-resistance. We therefore also measured the growth inhibitory activity of the trichothecenes against Adriamycin-resistant P388 cells. Furthermore, as resistance to Adriamycin could be circumvented by the coadministration of verapamil [9, 14], the restoration of sensitivity to T-2 toxin and some other trichothecenes by verapamil in drug-resistant cells was also attempted.

Materials and methods

T-2 toxin, 4,15-diacetylscirpenol, and neosolaniol were isolated from *Fusarium* sporotrichioides. Derivatives of T-2 toxin and 4,15-diacetylscirpenol were synthesized in our laboratory [12]. Verrucarin A and roridin A were obtained as a gift from Makor Co. (Jerusalem). All compounds were over 99% chemically pure; their structures were confirmed by nuclear magnetic resonance (NMR) and mass spectrometry.

P388 murine leukemia cells and a subline resistant to Adriamycin (P388/ADR) were continuously propagated in a suspension culture as previously described [10]. Briefly, the cells were maintained in RPMI 1640 (Biological Industries; Bet Haemek, Israel) medium supplemented with 10% fetal calf serum (Grand Island Biological Co.; Grand

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Abbreviations used: P388/ADR, Adriamycin-resistant P388 cells; ED_{50}, the concentration inhibiting the growth rate by 50%; P, octanol-water partitioning coefficient

Island, NY), 10 μ*M* 2-mercaptoethanol, penicillin free base (50 units/ml), and streptomycin (50 μg/ml). An inoculum of cells was transferred to fresh medium once every 4 days to maintain their exponential growth. Cell growth was assessed by measurements of cell density in a Coulter counter (Coulter Electronics; Harpenden, Hertfordshire, England). Cell growth rates were calculated from the culture densities measured once a day for 4 days.

Drug sensitivity was assessed as follows. Cells were cultured in the presence of various drug concentrations, and the slope of the log cell density vs time plot was calculated by linear regression analysis. The growth rate at each drug concentration was expressed as the percentage of the control growth rate. Dose-effect curves were thus produced and used to determine the concentration of drug effective in inhibiting the growth rate by 50% (ED_{50}). In repeated experiments, the standard deviation of this parameter was always $<10\%$ of the ED_{50} values obtained. The compounds were dissolved in ethanol. For each compound, the highest concentration tested was 6×10^{-5} *M* and the ethanol volume was $<0.6\%$ of the culture medium volume; this concentration of ethanol did not affect the growth rate of either cell line.

Results and discussion

As most compounds enter cells by passive diffusion through solubilization in the lipid matrix of the membrane, the lipophilic properties of each member of a series of compounds is frequently the single most important factor in determining biological activity [8]. From the number of esterified hydroxyl groups in verrucarol, scirpentriol, 4,15-diacetylscirpenol, and 3,4,15-triacetylscirpenol, Grove and Mortimer [5] concluded that the relationship between the lipoid solubility of these compounds and their growth inhibitory activity against HEp2 cells was only roughly positive. The chemical structures of the trichothecenes and their growth inhibitory activities against P388 and P388/ADR cells are contained in Table 1. Unfortunately, the octanol-water partitioning coefficients (P) of these trichothecenes were not available. However, it has been suggested that the relative hydrophobicity can be obtained from chromatographic R_f, values, since chromatography is largely a partitioning process [6]. Hansch [6] has pointed out that the term R_m, defined as

$$R_m = \log[(1/R_f)-1],$$

is linearly related to log P [12]. As thin-layer chromatography R_f values in toluene:ethyl acetate:formic acid (60:30:10 by volume) have been reported for most trichothecenes used in the present study [11], the relationship between their hydrophobicity and activity could be examined in both cell lines. As shown in Fig. 1, the R_m values of the trichothecenes with the strongest growth inhibitory activity were about 0.4, but there was no clear correlation between the degree of hydrophobicity of these compounds to their growth inhibitory activity against P388 cells. Similar results were obtained in P388/ADR cells (data not shown).

As shown in Table 1, the growth inhibitory effects of 4,15-diacetylscirpenol in Adriamycin-sensitive and -resistant P388 cells were comparable with those obtained using T-2 toxin. These results suggest that the isovaleric ester in position C-8 might be irrelevant to the activity. However,

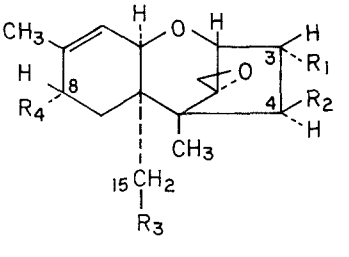
Table 1. Chemical structures and growth inhibitory activities of 12,13-epoxytrichothecenes in drug-sensitive and -resistant P388 cells

Name	R_1	R_2	R_3	R_4	ED_{50} (M)	
					P388	P388/ADR
1. T-2 toxin	OH	OAC	OAC	X	2.0×10^{-9}	2.0×10^{-8}
2. 3-Acetyl T-2 toxin	OAC	OAC	OAC	X	6.0×10^{-8}	2.0×10^{-7}
3. 3-Palmityl T-2 toxin	OPalmityl	OAC	OAC	X	3.0×10^{-7}	6.0×10^{-6}
4. Iso T-2 toxin	OAC	OH	OAC	X	1.5×10^{-7}	2.0×10^{-6}
5. HT-2 toxin	OH	OH	OAC	X	2.0×10^{-8}	3.0×10^{-7}
6. T-2 triol	OH	OH	OH	X	3.0×10^{-7}	6.0×10^{-6}
7. 9, 10-Dihydro T-2 toxin	OH	OAC	OAC	X	2.0×10^{-7}	4.5×10^{-7}
8. 9, 10-Epoxy T-2 toxin	OH	OAC	OAC	X	6.0×10^{-8}	8.0×10^{-7}
9. Neosolaniol	OH	OAC	OAC	OH	1.0×10^{-7}	2.0×10^{-6}
10. T-2 tetraol	OH	OH	OH	OH	4.5×10^{-7}	2.0×10^{-6}
11. 4, 15-Diacetylscirpenol	OH	OAC	OAC	H	4.5×10^{-9}	2.0×10^{-8}
12. 3, 4, 15-Triacetylscirpenol	OAC	OAC	OAC	H	8.0×10^{-8}	3.0×10^{-7}
13. 15-Monoacetylscirpenol	OH	OH	OAC	H	1.5×10^{-8}	1.2×10^{-7}
14. Scirpentriol	OH	OH	OH	H	5.0×10^{-8}	1.5×10^{-7}
15. Verrucarol	H	OH	OH	H	1.2×10^{-5}	2.0×10^{-5}
16. 4, 15-Diacetylverrucarol	H	OAC	OAC	H	4.5×10^{-7}	2.0×10^{-6}
17. Verrucarin A	H	O[a]	O[a]	H	6.0×10^{-9}	2.0×10^{-8}
18. Roridin A	H	O[b]	O[b]	H	1.3×10^{-9}	3.0×10^{-9}

OAC, $OOCCH_3$; X, $OOCCH_2CH(CH_3)_2$

[a] R_2 linked with R_3 through $OCCH(OH)CH(CH_3)CH_2CH_2OCOCH=CHCH=CHCO$

[b] R_2 linked with R_3 through $OCCH(OH)CH(CH_3)CH_2CH_2OCH(CHOCH_3)CH=CHCH=CHCO$



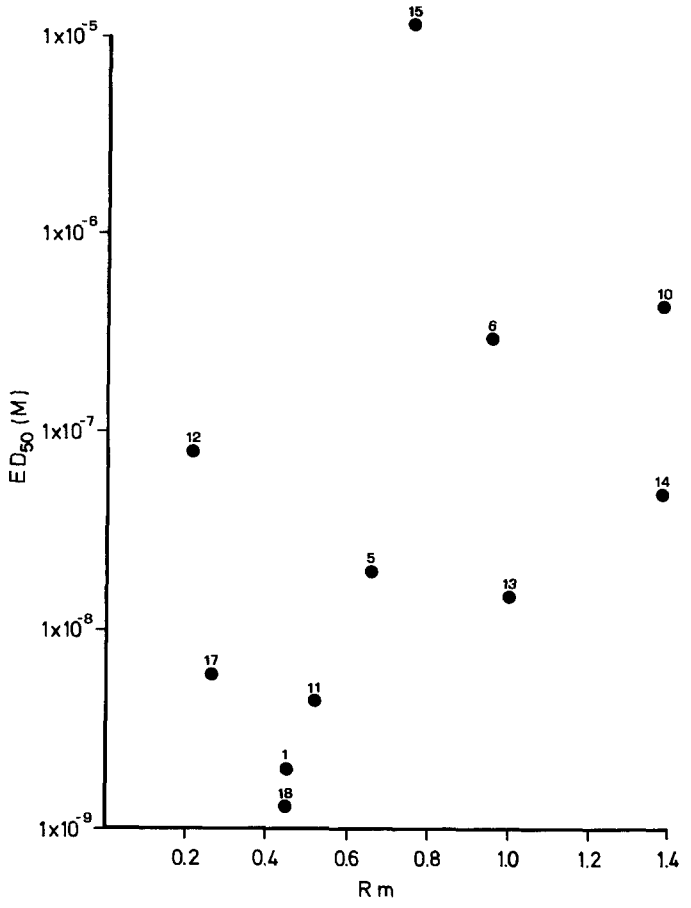


Fig. 1. The relationship between hydrophobicity and growth inhibitory activity of 12,13-epoxytrichothecenes in P388 cells. Hydrophobicity was calculated as R_m, as explained in the text. The *numbers above the dots* represent the trichothecene numbers as presented in Table 1

the considerably lower activity observed with neosolaniol indicates that a free hydroxyl group in the C-8 position is detrimental to the growth inhibitory activity. The 10-fold lower activity of T-2 tetraol in comparison with scirpentriol also supports this conclusion. We suggest that the masking of this free hydroxyl group by esterification results in enhanced growth inhibitory activity. The esterification of the hydroxyl in position C-8 of T-2 tetraol with isovaleric acid (resulting in T-2 triol), although resulting only a small improvement in activity, is a case in point.

In a previous study, the activity of 15-monoacetylscirpenol against P388 leukemia in vivo was found to be stronger than that of 3,15-diacetylscirpenol [1]. However, as 3-monoacetylscirpenol was as active as scirpentriol and 3,4-diacetylscirpenol was even more potent than 4-monoacetylscirpenol, the contribution of the hydroxyl group in position C-3 to the antitumor activity could not be determined. In the present in vitro study, 3-acetyl T-2 toxin was found to be 30-fold less cytotoxic to P388 cells than T-2 toxin, Iso T-2 toxin was 10-fold less active than HT-2 toxin, and 3,4,15-triacetylscirpenol was 18-fold less inhibitory than 4,15-diacetylscirpenol. These results indicate that the acetylation of the hydroxyl in the C-3 position results in considerable loss of activity. The esterification of this hydroxyl with palmitic acid (3-palmityl T-2 toxin) also resulted in a considerable decline of the growth inhibitory activity. The low inhibitory activity of verrucarol in comparison with that of scirpentriol and the ratio of growth inhibitory activity of 4,15-diacetylverrucarol to 4,15-diacetylscirpenol were further evidence for the contribution of a free hydroxyl function in the C-3 position to the growth inhibitory activity.

In Adriamycin-sensitive P388 cells, HT-2 toxin was 10-fold less cytotoxic than T-2 toxin and 15-monoacetylscirpenol had 3-fold less inhibitory activity than 4,15-diacetylscirpenol, indicating that hydrolysis of the acetyl ester in position C-4 could reduce the activity. However, it could not be determined as to whether the loss of the acetyl group or the presence of a free hydroxyl in position C-4 caused the reduction in activity. In contrast to these in vitro results, Doyle and Bradner [3] have reported that the potency of 15-monoacetylscirpenol against P388 cells in vivo was 2- to 4-fold higher than that of 4,15-diacetylscirpenol. These in vivo results led to the suggestion that the 15-monoacetylscirpenol could have been the active antitumor metabolite of 4,15-diacetylscirpenol. The result obtained with P388 cells in vitro are not compatible with such a mechanism; therefore, other explanations for the increased in vivo potency of 15-monoacetylscirpenol should be explored.

T-2 triol was found to be 15-fold less active than HT-2 toxin and scirpentriol was 3-fold less active than 15-monoacetylscirpenol, indicating that the hydrolysis of the acetyl group in position C-15 of the trichothecens was also detrimental to the cytotoxic activity. However, here again, it could not be determined as to whether this was due to the exposure of a free hydroxyl or the loss of the acetyl group. The results obtained with verrucarin A and roridin A could be interpreted as supporting the suggestion that esterification of the free hydroxyls in positions C-4 and C-15 enhanced the growth inhibitory activity. However, due to the intricate character of the diester bridges, and as only two macrocyclic derivatives were tested, such an interpretation should be viewed with caution. The results obtained with 9,10-dihydro and 9,10-epoxy analogues of T-2 toxin

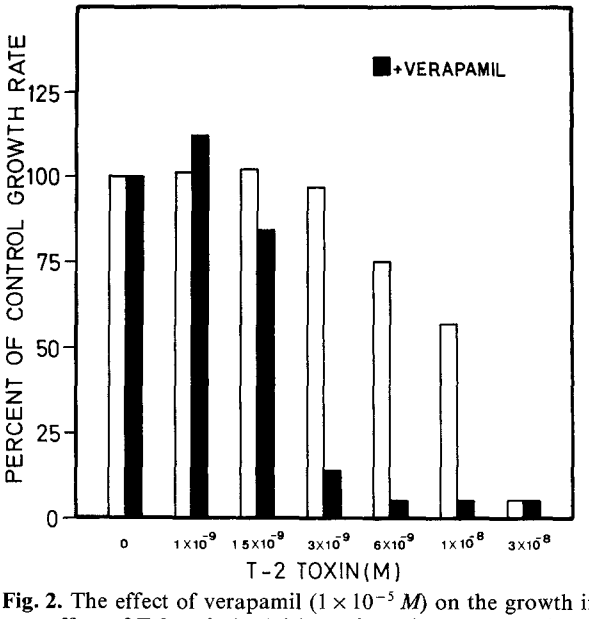


Fig. 2. The effect of verapamil (1×10^{-5} *M*) on the growth inhibitory effect of T-2 toxin in Adriamycin-resistant P388 cells

indicated as was previously suggested [3, 5], that the double bond in this location supported the growth inhibitory activity in both cell lines.

In the present study, we found that to obtain a growth inhibition in P388/ADR cells by the trichothecenes that would be comparable in magnitude to that obtained in Adriamycin-sensitive cells, a 1.7- to 20-fold higher concentration (depending on the derivative) was necessary. When all of the trichothecenes presented in Table 1 were lumped together, their P388/ADR to P388 ED_{50} ratio could not be correlated with their degree of hydrophobicity. However, in the group of 8-isovaleroxy derivatives containing hydroxyl and acetyl residues at positions C-3, C-4, and C-15, this ED_{50} ratio was found to be inversely related to the degree of hydrophobicity. We therefore suggest that other factors, as well as the hydrophobicity, determine the degree of cross-resistance of Adriamycin-resistant cells to the trichothecenes.

As shown in Fig. 2, the growth of P388/ADR cells in the absence of T-2 toxin was not affected by the presence of 1×10^{-5} *M* verapamil. However, in the presence of verapamil, the sensitivity of these cells to T-2 toxin was increased 10-fold. Verapamil was also found to lower the ED_{50} of 4,15-diacetylscirpenol in these cells from 2.0×10^{-8} *M* to 4.5×10^{-9} *M* and that of roridin A from 3.0×10^{-9} *M* to 8.0×10^{-10} *M* (data not shown). These results suggest that the mechanism of multidrug resistance is probably responsible for the resistance to the trichothecenes.

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