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Scytophycins, Novel Microfilament-depolymerizing Agents Which Circumvent P-Glycoprotein-mediated Multidrug Resistance[1]

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ABSTRACT

Cells demonstrating the multidrug resistance phenotype because of overexpression of P-glycoprotein, a drug efflux pump, are resistant to the cytotoxic effects of most natural product drugs. To determine if P-glycoprotein confers resistance to the syctophycins, a family of natural cytotoxic macrolides recently isolated from cyanobacteria of the family Scytonemataceae, we have characterized the effects of these compounds on drug-sensitive (SKOV3) and drug-resistant (SKVLB1) human ovarian carcinoma cells. While SKVLB1 cells demonstrated >150- and 10,000-fold decreases in sensitivity to Adriamycin and vinblastine, respectively, they were equally sensitive as SKOV3 cells to the antiproliferative effects of tolytoxin and certain related scytophycins. The SKVLB1 cells were 4- to 11-fold resistant to other scytophycins and were 14-fold resistant to cytochalasin B. Microfilaments in SKOV3 and SKVLB1 cells were depolymerized by similar concentrations of tolytoxin, while cytochalasin B was less potent toward SKVLB1 cells than SKOV3 cells. Both tolytoxin and cytochalasin B enhanced the cytotoxicity of vinblastine toward SKVLB1 cells; however, neither compound affected the sensitivity to Adriamycin or cisplatin. Verapamil markedly increased the accumulation of [^{3}H]vinblastine by SKVLB1 cells, while cytochalasin B caused only modest increases, and tolytoxin had no effect on [^{3}H]vinblastine accumulation. These results suggest that some of the scytophycins, including tolytoxin, are not subject to P-glycoprotein-mediated efflux from cells exhibiting multidrug resistance due to overexpression of this transport protein. These compounds may therefore be useful for killing drug-resistant tumor cells.

INTRODUCTION

Cancer chemotherapy programs often provide temporary clinical improvement; however, over time, the appearance of less easily treated cancer cells often results in the ultimate failure of chemotherapy. Tumor cells which survive the initial therapeutic attack often recover with increased resistance to both the original therapeutic agent and other seemingly unrelated drugs. This phenomenon has been termed MDR[3] (reviewed in Ref. 1 to 3). Mechanisms for acquired drug resistance include overexpression or activation of P-glycoprotein, overexpression of glutathione *S*-transferases, alterations in topoisomerase activities, and certain protective metabolic alterations.

The P-glycoprotein system appears to be a primary physiological mechanism of MDR and has been the subject of considerable biochemical characterization (reviewed in Ref. 1, 4, and 5). P-Glycoprotein is a M_r 170,000 to 200,000 transmembrane protein which is homologous with certain bacterial transport ATPases. P-Glycoprotein acts as an ATP-dependent drug efflux pump, actively removing a variety of structurally diverse cytotoxic natural products, including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, actinomycin D, taxol, and gramicidin D (reviewed in Refs. 1 to 5). Enhanced efflux of these compounds reduces their intracellular accumulation and so reduces their cytotoxicity. In contrast, P-glycoprotein does not extrude small, hydrophilic drugs such as cisplatin, fluorouracil, and mercaptopurine. Cross-linking studies have demonstrated the binding of ATP and several drug analogues to P-glycoprotein. Tumor cells from patients undergoing chemotherapy often demonstrate elevated P-glycoprotein expression and activity, indicating that this mechanism of MDR is clinically important (6–9). In addition, colon carcinomas, lung adenocarcinomas, and disseminated malignant melanomas often demonstrate increased expression of P-glycoprotein even in patients who have not yet received chemotherapy (6). Levels of P-glycoprotein expression are inversely correlated with clinical responsiveness to natural product anticancer drugs. Therefore, novel compounds which reverse drug resistance or which are toxic to resistant cells could be used to impair the expansion of these cells, and so may be of high therapeutic and economic value.

The scytophycins are a newly identified class of natural cytotoxins that have been isolated from cyanobacteria of the family Scytonemataceae (10, 11). We have demonstrated that these compounds act as potent microfilament-depolymerizing agents in intact cells and with purified actin (12). In experiments described herein, we have examined the antiproliferative effects of the scytophycins and of cytochalasin B on drug-sensitive (SKOV3) and P-glycoprotein-overexpressing drug-resistant (SKVLB1) human ovarian carcinoma cells.

MATERIALS AND METHODS

Materials. Adriamycin, vinblastine, cytochalasin B, cisplatin, and sulforhodamine B were obtained from the Sigma Chemical Company. Tolytoxin and related scytophycins (Fig. 1) were purified from ethanol extracts of *Scytonema ocellatum* and *Scytonema burmanicum* as described by Carmeli *et al.* (11). These compounds were dissolved in ethanol and were stored at −20°. Concentrations of the scytophycins were determined using extinction coefficients defined by Carmeli *et al.* (11). BME was from Gibco, and fetal calf serum was purchased from Hyclone. [^{3}H]Vinblastine sulfate was a product of Amersham.

Cell Lines. Human ovarian carcinoma cells (SKOV3) and a subline which has been selected for resistance to vinblastine (SKVLB1) were a generous gift from Dr. Victor Ling of the Ontario Cancer Institute (13). Both cell lines were maintained in BME containing 10% fetal calf serum and 50 μg/ml of gentamycin sulfate. Vinblastine was added to a final concentration of 1 μg/ml to SKVLB1 cells 24 h after passage to maintain selection pressure for P-glycoprotein-overexpressing cells.

Cytotoxicity Assay. Cells were plated into 24-well tissue culture plates to a density of approximately 10% of confluency. After 24 h, the indicated concentrations of the cytotoxic agent were added, and the cells were incubated for an additional 48 h. Control cultures included equivalent amounts of the solvent, *i.e.*, ethanol or dimethyl sulfoxide, which did not modulate the growth or drug sensitivity of either cell line. Following the 48-h incubation, the medium was removed from each well, and cells were washed with PBS and then fixed with 10% trichloroacetic acid. The fixed cells were stained with 0.4% SRB in 1% acetic acid for 30 min as described by Skehan *et al.* (14). Unbound SRB was removed by four rapid washes with 1% acetic acid, and the bound SRB was solubilized in 1 ml of 10 mM unbuffered Tris base. The amount of bound SRB was determined by its absorbance at 560 nm. The percentage of cells killed by the cytotoxin was calculated as the percentage decrease in SRB binding compared with untreated cultures.

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[3] The abbreviations used are: MDR, multidrug resistance; BME, Eagle's basal medium; PBS, phosphate-buffered saline; SRB, sulforhodamine B; IC_{50}, concentration required to inhibit response by 50%; TRITC-phalloidin, phalloidin conjugated with tertamethylrhodamine isothiocyanate.

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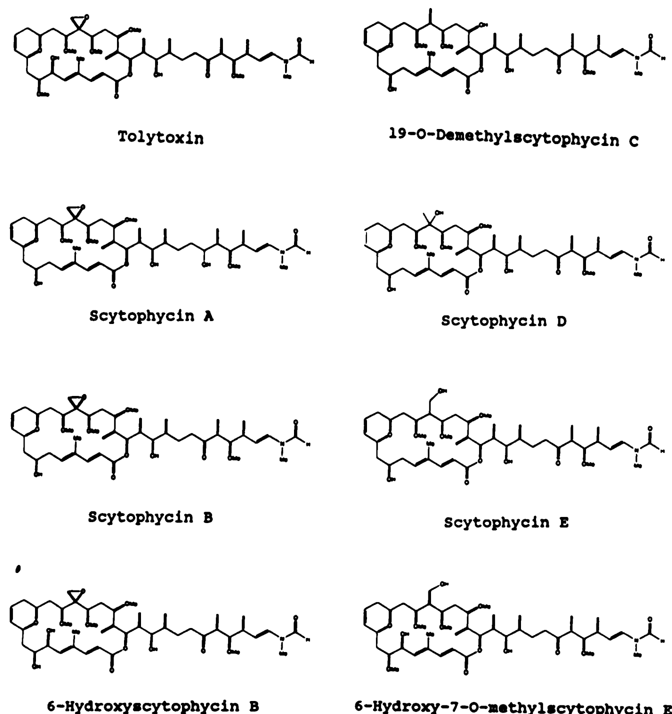

Fig. 1. Structures of the scytophycins.

In some experiments, cell proliferation was measured as the rate of incorporation of [^{3}H]thymidine into DNA (15) and by direct cell counting using a Coulter Counter. The percentage of cells killed was calculated as the percentage decrease in [^{3}H]thymidine incorporation or in cell number compared with untreated cultures.

Assay for Reversal of Multidrug Resistance. SKVLB1 cells were plated in 24-well dishes as described above. After 24 h, the cells were exposed to a range of concentrations of Adriamycin, vinblastine, or cisplatin in combination with buffer, verapamil, tolytoxin, or cytochalasin B. The cytotoxicities of these treatments were determined as described above.

[^{3}H]Vinblastine Accumulation Assay. SKVLB1 cells were plated into 24-well tissue culture dishes and allowed to grow to 90% of confluency. The cells were washed with PBS and then incubated in 0.5 ml of BME containing 15 nM [^{3}H]vinblastine sulfate (11.7 Ci/mmol) for 60 min at 37°C. The cultures were rapidly washed 3 times with cold PBS. Intracellular [^{3}H]vinblastine was solubilized with 1% Triton X-100 in PBS and quantitated by liquid scintillation counting.

Quantitation of f-Actin. SKOV3 or SKULB1 cells were grown to confluency in 24-well tissue culture plates as described above. Cytochalasin B or tolytoxin was diluted in PBS and incubated with triplicate samples of cells for 24 h. Cells were then fixed with para-formaldehyde and permeabilized with Triton X-100 as described above. Aliquots (300 μl) of PBS containing 250 nM TRITC-phalloidin were added to each well and allowed to incubate for 60 min at 37°C. The wells were rapidly washed with cold PBS, and bound TRITC-phalloidin was solubilized with 0.8 ml of 1% sodium dodecyl sulfate. The fluorescence of the samples was quantitated using an excitation wavelength of 530 nm and an emission wavelength of 560 nm. The amount of bound TRITC-phalloidin never exceeded 10% of the total amount of TRITC-phalloidin in the incubations. Nonspecific binding of TRITC-phalloidin was determined in samples containing 500 nM phalloidin (Sigma) and did not exceed 5% of the total binding to untreated cells.

RESULTS

Cytotoxicities of Scytophycins. The antiproliferative effects of Adriamycin, vinblastine, cytochalasin B, cisplatin, and several scytophycins were determined using both drug-sensitive SKOV3 cells and drug-resistant SKVLB1 cells. As indicated in Table 1, the SKVLB1 cells are markedly (10,000-fold) resistant to the cytotoxic actions of vinblastine compared with SKOV3 cells. The SKVLB1 cells also demonstrate strong resistance to Adriamycin, but are as sensitive to cisplatin as are the parental SKOV3 cells. Cytochalasin B also has a reduced efficacy for the inhibition of SKVLB1 cell growth, suggesting that overexpression of P-glycoprotein confers resistance to this microfilament-depolymerizing agent. In contrast, the two cell lines demonstrate essentially equal sensitivities to the antiproliferative effects of tolytoxin, scytophycin A, and scytophycin B. The SKVLB1 cells demonstrated modest resistance (approximately 4-fold) to 6-hydroxyscytophycin B and 19-*O*-demethylscytophycin C, as well as approximately 8-fold resistance to scytophycin D, scytophycin E, and 6-hydroxy-7-*O*-methylscytophycin E. The scytophycins are less potent for the inhibition of SKOV3 cell growth than is vinblastine; however, these compounds are much more potent cytotoxins than Adriamycin, cytochalasin B, and cisplatin.

The effects of tolytoxin on the growth of SKOV3 and SKVLB1 cells were characterized using three different assays for cell growth. As demonstrated in Fig. 2, tolytoxin caused dose-dependent decreases in growth when measured using the SRB-binding assay, [^{3}H]thymidine incorporation into DNA, or by direct cell counting. The IC_{50} for

Table 1 *Cytotoxicities of scytophycins toward SKOV3 and SKVLB1 cells*

SKOV3 and SKVLB1 cells were treated with increasing concentrations of the indicated compounds for 48 h as described in "Materials and Methods." Cell numbers were then determined by protein staining with sulforhodamine B. Values represent the concentration of compound required to decrease the cell number by 50% compared with untreated cultures. Fold-resistance values are calculated as the ratio of IC_{50}s for SKVLB1 and SKOV3 cells. The means of three experiments are indicated.

Compound	IC_{50} (nM) SKOV3	SKVLB1	Fold-resistance
Adriamycin	730 ± 144	>100,000	>136
Vinblastine	1.0 ± 0.2	10,000 ± 1,000	10,000
Cytochalasin B	4,000 ± 800	55,000 ± 13,000	13.8
Cisplatin	2,000 ± 140	2,000 ± 112	1.0
Tolytoxin	23 ± 3	37 ± 5	1.6
Scytophycin A	53 ± 19	92 ± 12	1.7
Scytophycin B	83 ± 27	83 ± 27	1.0
6-OH-Scytophycin B	48 ± 15	188 ± 20	3.9
19-*O*-Demethylscytophycin C	25 ± 2	110 ± 22	4.4
Scytophycin D	133 ± 24	1,000 ± 89	7.5
Scytophycin E	90 ± 11	1,000 ± 120	11.1
6-OH-7-*O*-Methylscytophycin E	108 ± 26	>1,000	>9.3

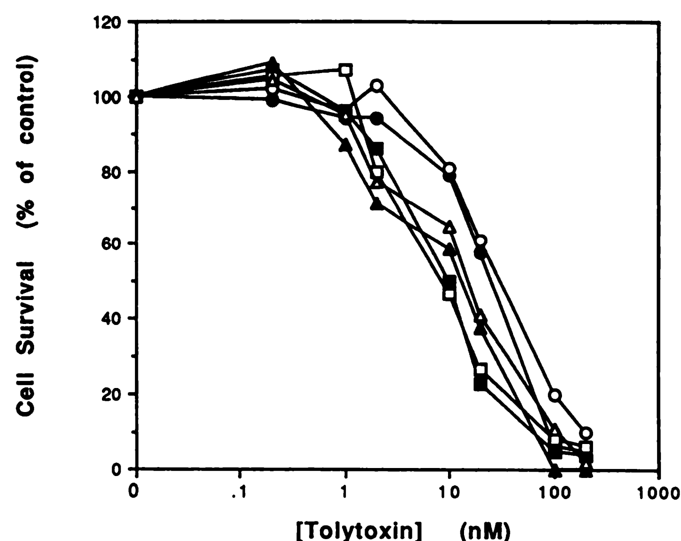

Fig. 2. Cytotoxicities of tolytoxin for SKOV3 and SKVLB1 cells. SKOV3 cells (*filled symbols*) and SKVLB1 cells (*open symbols*) were treated for 48 h with the indicated concentrations of tolytoxin. Cell survival was determined as described in "Materials and Methods" using the SRB assay (*circles*), the [^{3}H]thymidine incorporation assay (*squares*), or by cell counting (*triangles*). *Points*, mean of triplicate samples in one of three experiments. SDs averaged less than 6% for all three assays.

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tolytoxin varied by approximately 2-fold in these assays; however, SKOV3 and SKVLB1 cells demonstrated equal sensitivities to tolytoxin in all three assay systems. Tolytoxin caused pronounced morphological changes in the cells, characterized initially by cell contraction and rounding followed by blebbing, but did not induce the detachment of either type of cell from the tissue culture plates (data not shown).

Chemosensitizing Effects of Tolytoxin and Cytochalasin B. Chemosensitizing agents potentiate the cytotoxicity of natural product anticancer drugs in SKVLB1 and other MDR cells. Verapamil is well known to interact with P-glycoprotein and markedly potentiated the ability of vinblastine (Fig. 3*A*) and Adriamycin (Fig. 3*B*) to kill SKVLB1 cells. In contrast, verapamil did not modulate the toxicity of cisplatin (Fig. 3*C*), which is not subject to P-glycoprotein-mediated export. Neither tolytoxin nor cytochalasin B modulated the cytotoxicities of Adriamycin and cisplatin (Fig. 3, *B* and *C*); however, both compounds enhanced the cytotoxicity of vinblastine (Fig. 3*A*). Isobologram analyses (Fig. 4) demonstrated that all tested doses of tolytoxin and cytochalasin B promote greater than additive inhibition of cell proliferation in combination with vinblastine. Tolytoxin was approximately 100-fold more potent than cytochalasin B in enhancing the toxicity of vinblastine.

Effects of Cytochalasin B and Tolytoxin on [^{3}H]Vinblastine Accumulation by SKVLB1 Cells. Since P-glycoprotein outwardly transports natural product drugs, increased accumulation of intracellular [^{3}H]vinblastine by MDR cells is indicative of inhibition of P-glycoprotein activity. As expected, verapamil caused dose-dependent increases in [^{3}H]vinblastine accumulation by SKVLB1 cells (Fig. 5). The doses of verapamil required for this effect were similar to those required for chemosensitization of the cells to vinblastine and Adriamycin, indicating a good correlation between cytotoxicity and competitive inhibition of P-glycoprotein activity. Cytochalasin B also enhanced [^{3}H]vinblastine accumulation at doses of 10 μM and greater. These concentrations of cytochalasin B are well below the IC_{50} for SKVLB1 cell proliferation and are similar to doses required for the enhancement of vinblastine cytotoxicity. In contrast, tolytoxin did not modulate [^{3}H]vinblastine accumulation by these cells, even at doses which enhance the cytotoxicity of vinblastine (*i.e.* 10 nM).

Effects of Cytochalasin B and Tolytoxin on f-Actin Levels in SKOV3 and SKVLB1 Cells. The effects of cytochalasin B and tolytoxin on levels of f-actin in intact cells were determined by measuring the binding of TRITC-phalloidin to cells fixed and permeabilized after exposure to the drugs. As demonstrated by Fig. 6, both cytochalasin B and tolytoxin caused dose-dependent decreases in binding of TRITC-phalloidin to the permeabilized cells. The 50% effective concentrations for tolytoxin and cytochalasin B for decreased binding of TRITC-phalloidin to SKOV3 cells were approximately 10 nM and 1 μM, respectively, and closely correspond to doses of these drugs needed to observe stress fiber disruption by fluorescence microscopy (data not shown). SKVLB1 cells were approximately 10-fold resistant to microfilament disruption by cytochalasin B, but were equally sensitive as SKOV3 cells to tolytoxin. Neither tolytoxin nor cytochalasin B caused detachment of the cells from the culture plates as determined by both cell counting and protein quantitation (data not shown). Binding of TRITC-phalloidin to permeabilized cells was strongly inhibited by phalloidin, but was not affected by doses of tolytoxin up to at least 1 μM or cytochalasin B at doses up to at least 100 μM.

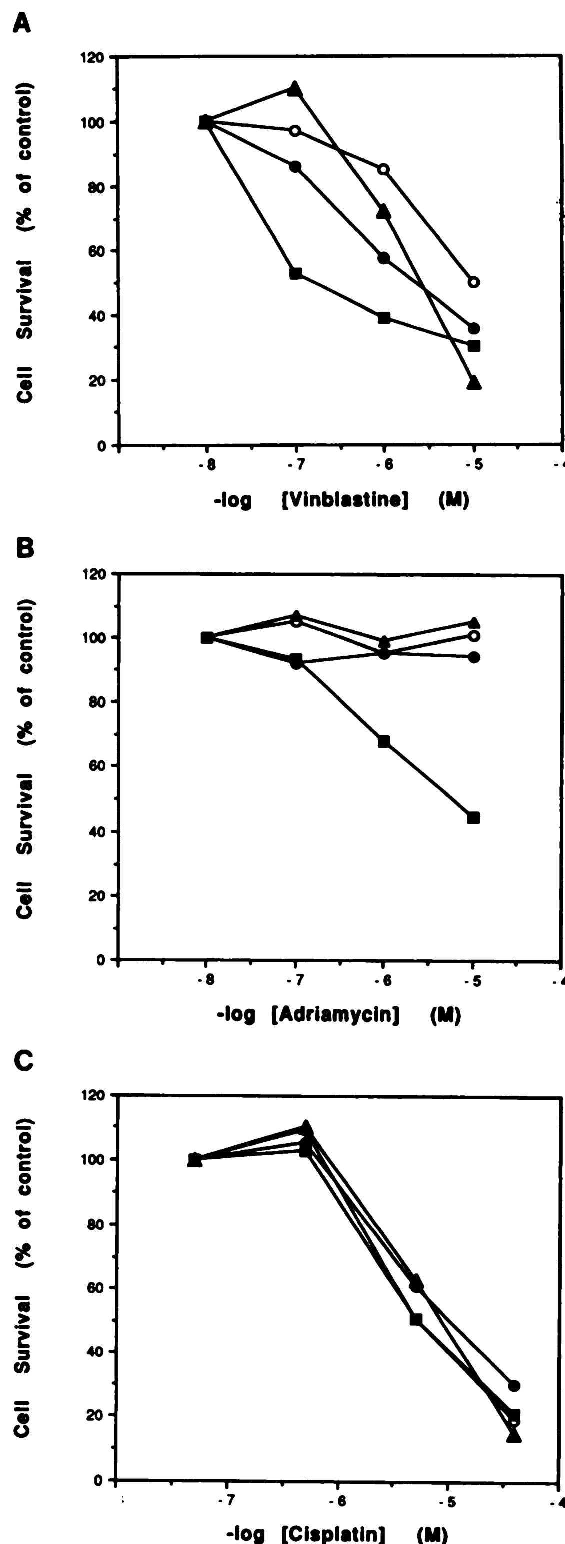

Fig. 3. Chemosensitization by verapamil, tolytoxin, and cytochalasin B. SKVLB1 cells were treated for 48 h with the indicated concentrations of vinblastine (*A*), Adriamycin (*B*), or cisplatin (*C*) in the presence of the carrier solvent dimethylformamide (○), 20 μM verapamil (■), 10 nM tolytoxin (▲), or 20 μM cytochalasin B (●). Cell survival was determined using the SRB binding assay. Points, mean of triplicate cultures in one of three experiments.

DISCUSSION

The scytophycins are a family of newly identified cytotoxins isolated from cyanobacteria. We have recently shown that these compounds have marked effects on cell morphology, inducing surface blebbing and polynucleation. These effects appear to be caused by selective disruption of microfilaments in scytophycin-treated cells (12). Microfilament depolymerization by these compounds occurs in the absence of alterations in the distribution of either microtubules or

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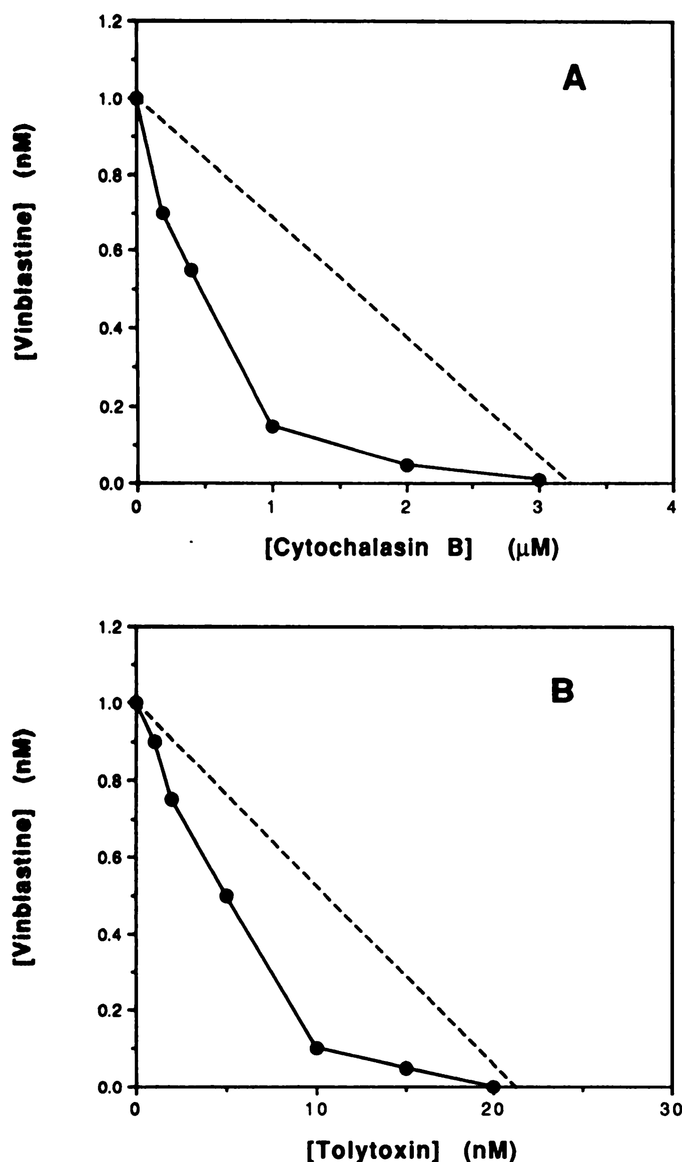

Fig. 4. Isobolo analysis of the cytotoxicities of vinblastine in combination with cytochalasin B and tolytoxin. SKOV3 cells were treated with vinblastine (ranging from 0 to 2 nM) and cytochalasin B at the doses indicated in *A* or tolytoxin at the doses indicated in *B* for 48 h. Cell survival was measured using the SRB binding assay as described in "Materials and Methods." Doses of vinblastine required for 50% inhibition of growth were then determined and are indicated (●). The line of additivity (– – – –) extends from the IC_{50}s for vinblastine and cytochalasin B or tolytoxin. Similar results were obtained in three experiments.

intermediate filaments. Therefore, the scytophycins appear to have cytoskeletal and morphological effects very similar to the cytochalasins. However, the scytophycins are 100- to 1000-fold more potent than cytochalasin B in inducing microfilament depolymerization and cytotoxicity. Because of the potent cytotoxicities of the scytophycins and the general lack of efficacy of anticancer drugs toward cells which have developed P-glycoprotein-mediated MDR, we have characterized the effects of these new cytotoxins on human ovarian carcinoma cells.

We have compared the cytoxicities of vinblastine and Adriamycin, which are known to be substrates for excretion by P-glycoprotein, with responses to scytophycins and cytochalasin B. Resistance of the SKVLB1 cells to these agents is considered to indicate the ability of P-glycoprotein to outwardly transport the toxin. Comparison of the IC_{50}s of the scytophycins for SKOV3 and SKVLB1 cells (Table 1) indicates that SKVLB1 cells demonstrate differential resistance to these compounds. No or only limited resistance is manifested toward scytophycins A and B and tolytoxin, while scytophycins D and E and 6-OH-7-*O*-methylscytophycin E are significantly less toxic toward SKVLB1 cells than SKOV3 cells. The primary structural difference between these two sets of compounds is the presence of an epoxide at C-16 of scytophycins A and B. This feature appears to overcome the ability of P-glycoprotein to protect cells from the actions of these cytotoxins. It is also interesting to note that, while SKVLB1 cells are not resistant to scytophycin B and tolytoxin (6-OH-7-*O*-methylscytophycin B), these cells are approximately 4-fold less sensitive to 6-OH-scytophycin B. Therefore, moieties at positions C-6 and C-7 also

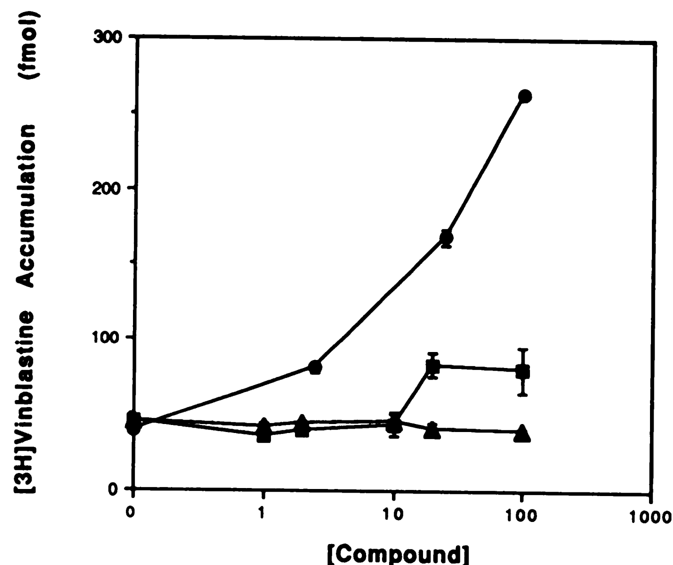

Fig. 5. Effects of verapamil, tolytoxin, and cytochalasin B on [^{3}H]vinblastine accumulation by SKVLB1 cells. SKVLB1 cells at approximately 90% of confluency were incubated with the indicated concentrations of verapamil (●), cytochalasin B (■), or tolytoxin (▲) for 60 min before the addition of 16 nM [^{3}H]vinblastine. Cultures were further incubated for 60 min before the levels of intracellular [^{3}H]vinblastine were determined. Drug concentrations for verapamil and cytochalasin B are μM, while those of tolytoxin are nM. *Points*, mean for triplicate cultures; *bars*, SD.

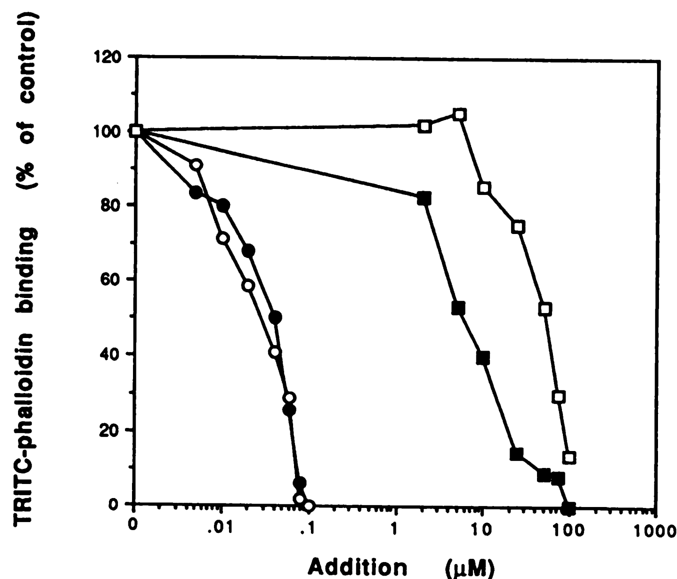

Fig. 6. Effects of tolytoxin and cytochalasin B on f-actin levels in SKOV3 and SKVLB1 cells. SKOV3 cells (*filled symbols*) or SKVLB1 cells (*open symbols*) were treated with the indicated concentrations of tolytoxin (*circles*) or cytochalasin B (*squares*) for 24 h. The cells were then fixed, permeabilized, and incubated with TRITC-phalloidin as described in "Materials and Methods." The amount of bound TRITC-phalloidin is expressed as the percentage of that bound by cells incubated in the absence of either tolytoxin or cytochalasin B. *Points*, mean ± SEM for three experiments.

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appear to influence the interaction of scytophycins with P-glycoprotein. We are currently characterizing the abilities of these scytophycins to bind to P-glycoprotein.

Additional studies (not shown) have demonstrated that Adriamycin-resistant HL-60 cells and Adriamycin-resistant MCF-7 cells, both of which overexpress P-glycoprotein, are also not resistant to scytophycins. Therefore, the effects described here are not limited to the ovarian carcinoma cell line.

Both tolytoxin and cytochalasin B enhance the cytotoxicity of vinblastine toward SKVLB1 cells. Tsuruo and Iida have reported that cytochalasins may inhibit P-glycoprotein activity since they enhanced daunomycin and vincristine accumulation in P-glycoprotein-overexpressing cells (16). However, in our studies, these effects do not appear to be mediated by inhibition of P-glycoprotein function, since neither compound potentiates the cytotoxicity of Adriamycin. In contrast, verapamil, which is well accepted as an inhibitor of P-glycoprotein function because of its ability to compete for binding to the transport protein, chemosensitizes the SKVLB1 cells to both vinblastine and Adriamycin. Tolytoxin, cytochalasin B, and verapamil all have negligible effects on the cytotoxicity of cisplatin which is not subject to export by P-glycoprotein. Tolytoxin and cytochalasin B cause similar potentiation of vinblastine toxicity toward SKOV3 cells, further suggesting that this effect is not mediated by inhibition of P-glycoprotein. The synergistic interactions between vinblastine and cytochalasin B or tolytoxin may be mediated by disruption of independent cytoskeletal structures, *i.e.*, microtubules and microfilaments, respectively. Loss of both types of intracellular structures may impair cell functions such as proliferation and attachment more strongly than the loss of either cytoskeletal component alone.

Recent studies have suggested that cytochalasin B reduces *in vivo* tumor growth and metastasis (17). Our studies also demonstrate that overexpression of P-glycoprotein confers resistance to cytochalasin B-induced microfilament depolymerization and inhibition of cell growth. Cytochalasin B also causes modest increases in the accumulation of [^{3}H]vinblastine in SKVLB1 cells, suggesting that it may weakly compete for binding to P-glycoprotein. Additionally, accumulation of [^{3}H]cytochalasin B in SKVLB1 cells is approximately 3-fold lower than in SKOV3 cells. Verapamil markedly increases the accumulation of [^{3}H]cytochalasin B in SKVLB cells, but not in SKOV3 cells.[4] Therefore, cytochalasin B would not be expected to be useful for the destruction of tumor cells which have acquired MDR via P-glycoprotein, and so may be of limited usefulness as an anticancer drug. In contrast, since certain scytophycins such as tolytoxin and scytophycin B demonstrate equal efficacy toward drug-sensitive and drug-resistant cells, these agents may be useful in the therapy of resistant tumors. Additionally, since these compounds appear not to interact with P-glycoprotein, their use in chemotherapy may avoid the induction of P-glycoprotein. Only a few structurally complex drugs, *e.g.*, gossypol (18), MX2 (19), spirogermanium (20), NC-190 (21), and NSC 370147 (22), share this property of avoidance of P-glycoprotein-mediated export. Therefore, the scytophycins represent a new family of compounds that may be very useful in the treatment of cancer.

[4] Manuscript in preparation.

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