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ORIGINAL ARTICLE

Julie A. Holmes · Peter R. Twentyman

The activity of deoxyspergualin in multidrug-resistant cells

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Abstract Deoxyspergualin, a synthetic analogue of the immunosuppressive anti-tumour antibiotic spergualin, has been shown to possess potent in vitro and in vivo anti-tumour activity and is currently in the National Cancer Institute (NCI) decision network. Deoxyspergualin shows similarities in properties and mechanisms of action to the natural-product immunosuppressive agents cyclosporin A and FK506, each of which can act as a modifier of multidrug resistance. We therefore decided to examine the comparative activity of deoxyspergualin in parent and multidrug-resistant cells. Deoxyspergualin contains the polyamine spermidine within its structure. Bovine serum copper amine oxidase catalyses the oxidative deamination of spermidine to produce an aminoaldehyde, ammonia and hydrogen peroxide. These aminoaldehydes are believed to be responsible for the toxicity of polyamines in vitro in the presence of bovine serum. For this reason, all experiments were carried out in medium containing bovine serum and in medium containing horse serum (which is low in copper amine oxidase content). We used the tetrazolium (MTT) colorimetric assay to determine drug sensitivity and tritiated daunorubicin accumulation together with inhibition of azidopine binding to study specific mechanisms of resistance modulation. The murine cell lines EMT6/P and EMT6/AR1.0 and the human cell lines H69/P and H69/LX4 were, respectively, 32-, 32-, 372- and 483-fold more sensitive to spermidine and 175-, 133-, 321- and 444-fold more sensitive to spermine in the presence of calf serum than in the presence of horse serum. However, these large differential effects were not seen for deoxyspergualin. It appears that in the presence of horse serum, deoxyspergualin may exert its effect by a mechanism other than polyamine oxidation. Deoxyspergualin did not enhance the accumulation of [^{3}H]-daunorubicin in EMT6/AR1.0 cells. Furthermore, deoxyspergualin (1–20 μM) did not restore the sensitivity of EMT6/AR1.0 or H69/LX4 cells to that of the parent lines. P-glycoprotein (Pgp) in membranes prepared from H69/LX4 cells was photo-affinity-labeled with [^{3}H]-azidopine. Deoxyspergualin did not inhibit this labeling. Although deoxyspergualin appears to exert its immunosuppressive effect via a mechanism similar to that of cyclosporin A and FK506, it does not share their ability to modify Pgp-mediated multidrug resistance. However, its lack of cross-resistance and potent in vivo anti-tumour activity make deoxyspergualin a promising candidate for development as an anti-cancer agent.

Key words Multidrug resistance · Deoxyspergualin · Polyamine oxidation

J. A. Holmes · P. R. Twentyman (✉)
MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge CB2 2QH, UK

Introduction

Deoxyspergualin is a synthetic derivative of the anti-tumour antibiotic spergualin produced by a strain of *Bacillus laterosporus* [16, 22]. Spergualin has good anti-tumour activity against murine neoplasms, including lymphatic leukaemias L1210 and P388, mastocytoma P815, thymoma EL-4, sarcoma 180, and myeloid leukaemia 1498 [16]. Deoxyspergualin, currently in the National Cancer Institute (NCI) decision network, is more potent than spergualin in vivo and its direct synthesis is simpler; therefore it was selected as a candidate for further development. In addition to its anti-tumour activity, deoxyspergualin has also been shown to posses immunosuppressive activity in both in vitro and in vivo systems [11, 15]. The mechanism of immunosuppression of the drug is at present unclear; however, deoxyspergualin has been shown to increase interleukin 2 production in mixed lymphocyte culture, to enhance natural killer cell activity in spleen cells of tumour-bearing mice and to activate T-lymphocytes [4].

Cyclosporin A and FK506 are also natural products possessing potent immunosuppressive properties [15]. Deoxyspergualin has been shown to bind to the human constitutive heat-shock protein Hsp70 [10]. Cyclophilin, the cyclosporin A-binding protein, FK506-binding protein, and

the heat-shock proteins are involved in the regulation of protein folding. This factor and the ability of all three agents to inhibit the function of T-cell lymphocytes may suggest a common mechanism of action for these immunosuppressants.

Cells treated in vitro with one of a well-defined group of (mainly natural-product) cytotoxic agents acquire resistance to that agent and become simultaneously resistant to many other drugs that are structurally and functionally unrelated to the selecting drug [2, 3]. Drugs involved include doxorubicin, vincristine, etoposide and taxol. These cells are said to possess a multidrug-resistant phenotype and frequently hyperexpress a 170-kDa membrane glycoprotein (P-glycoprotein) that is believed to act as a drug-efflux pump [3]. This type of resistance is often referred to as 'classical multidrug resistance'.

Both FK506 and cyclosporin A have been shown to act as effective modifiers of classic multidrug resistance in that they can selectively restore sensitivity to the cells with acquired resistance [5, 18]. Deoxyspergualin is similar to cyclosporin A and FK506 in its properties and mechanism of action, and multidrug-resistant cells are cross-resistant to cyclosporin A. We therefore decided to address the following questions. Does deoxyspergualin act as a modifier of P-glycoprotein-mediated multidrug resistance. Are multidrug-resistant cells cross-resistant to deoxyspergualin?

The structure of the polyamine spermidine is present within the larger structure of deoxyspergualin, which can therefore be considered to be a spermidine analogue. Bovine serum copper amine oxidase catalyses the oxidative deamination of spermidine to produce an aminoaldehyde, ammonia and hydrogen peroxide. It is thought that these aminoaldehydes are responsible for the toxicity of polyamines in vitro in the presence of bovine serum [7, 13], whereas other mechanisms may predominate in this cytotoxicity under conditions where other serum types, low in copper amine oxidase content, are used. For this reason, all experiments involving the incubation of cells in medium were performed in duplicate using both bovine serum and horse serum, which is low in copper amine oxidase content [6].

Materials and methods

Cell lines

We used the mouse mammary-carcinoma cell line EMT6/Ca /VJAC, henceforth referred to as EMT6/P [14, 17], and the human small-cell lung-cancer cell line NCI-H69, henceforth referred to as H69/P, together with their P-glycoprotein hyper-expressing sublines EMT6/AR1.0 [21] and H69/LX4 [20]. EMT6 cells grow as attached monolayers in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with glutamine (25 μ*M*), penicillin (100 U/ml), streptomycin (100 μg/ml) and either 20% new-born calf serum (Sigma) or 20% horse serum (Sigma). They were disaggregated using a solution of trypsin (0.1%; Life Technologies) in phosphate-buffered saline (PBS). NCI-H69 cells were originally supplied by Dr. D. Carney and Dr. A. Gazdar of the NCI/Navy Medical Oncology Branch. This line grows as floating aggregates of cells in RPMI 1640 medium supplemented with gluatmine (25 μ*M*), penicillin (100 U/ml), streptomycin (100 μg/ml) and either 10% foetal calf serum or 10% horse serum (Sigma). All cells were maintained as stock cultures in plastic tissue-culture flasks, incubated at 37 °C in an atmosphere of 8% CO_2 and 92% air.

Drugs

Deoxyspergualin was kindly provided by the Drug Development Branch of the NCI (Bethesda, Md., USA). It was dissolved in sterile water at a concentration of 50 m*M* and aliquots were stored at –20 °C. Spermine and spermidine were obtained from Sigma and then dissolved in sterile water at a concentration of 500 mg/ml and stored at –20 °C. Cyclosporin A (Sandoz, Basel) was dissolved in absolute ethanol at 4.2 m*M* and stored at 4 °C. These drugs were diluted in medium immediately before each experiment. Appropriate solvent controls were used in all experiments. Solvent concentrations did not exceed 0.1%. Doxorubicin (Farmitalia) was dissolved at a concentration of 500 μg/ml in sterile water and aliquots were stored at –20 °C. Dilution in PBS was carried out immediately before use. Tritiated daunorubicin (2.6 Ci/mmol) was obtained from New England Nuclear and stored at –70 °C.

Drug sensitivity testing

The cytotoxicity of deoxyspergualin and the polyamines spermidine and spermine were determined using the tetrazolium (MTT) reduction colorimetric assay [9] as adapted for use in this laboratory [19]. Single-cell suspensions were prepared from exponentially growing cultures and inoculated into wells on 96-well tissue-culture plates in a volume of 200 μl/well at 3×10^3, 6×10^3, 3×10^4 and 5×10^4 cells/ml for EMT6/P, EMT6/AR1.0, H69/P and H69/LX4 cells, respectively. Cytotoxic agents were added 1 h later in a volume of 20 μl. After an incubation period of 3 days (EMT6) and 6 days (H69), 20 μl of a 5-mg/ml solution of MTT (Sigma) in PBS was added to each well. These times were chosen such that untreated cells of each of the cell lines increased by 10- to 20-fold in number during the incubation period. The plates were re-incubated for 5 h. At the end of this period the medium was aspirated from each well, 200 μl of dimethylsulphoxide was added and the plates were shaken on a plate shaker for 10 min. The optical desities of the wells were read on a Titretek Multiskan MCC/340 plate reader at a wavelength of 540 nm and at a reference wavelength of 690 nm. For resistance-modification assays the above-mentioned protocol was followed with the additional step of adding the modulating agent 1 h after the cells had been plated and 1 h before the addition of the drug. In all experiments each drug dose was studied in four replicate wells and the variation in final absorbance between replicate wells was generally less than 10%.

Clonogenic survival assays were also carried out on EMT6/P and EMT6/AR1.0 cells exposed to increasing concentrations of deoxyspergualin. Cells in drug-containing medium were plated onto 9-cm petri dishes (Nunc). They were incubated at 37 °C in a humidified atmosphere of 8% CO_2 in air for 9 days. At the end of this incubation period the medium was removed and the plates were rinsed in saline and then fixed and stained simultaneously in a solution of 5% crystal violet (Gurr) in 95% alcohol. Colonies were visualised with the aid of an Olympus stereo zoom microscope, and those with more than 50 cells were counted.

Drug accumulation studies

The effect of deoxyspergualin on the ability of EMT6/P and EMT6/AR1.0 cells to accumulate [^{3}H]-daunorubicin was determined as described previously [23]. Briefly, 48 h before the experiments, cells were inoculated into six-well plates at 2×10^4/well for EMT6/P and 4×10^4 for EMT6/AR1.0 in a volume of 2 ml. To commence experiments, medium was aspirated from the wells and replaced with medium containing [^{3}H]-daunorubicin (0.1 μCi/ml) together with unlabeled drug to give a final concentration of 1 μ*M* and, where appropriate, deoxyspergualin at varying concentrations. As a positive control the accumulation study was also carried out in the presence of 1 μ*M* cyclosporin A. The plates were then incubated for a period of 1 h. At the end of this time the wells were rinsed three times with ice-cold

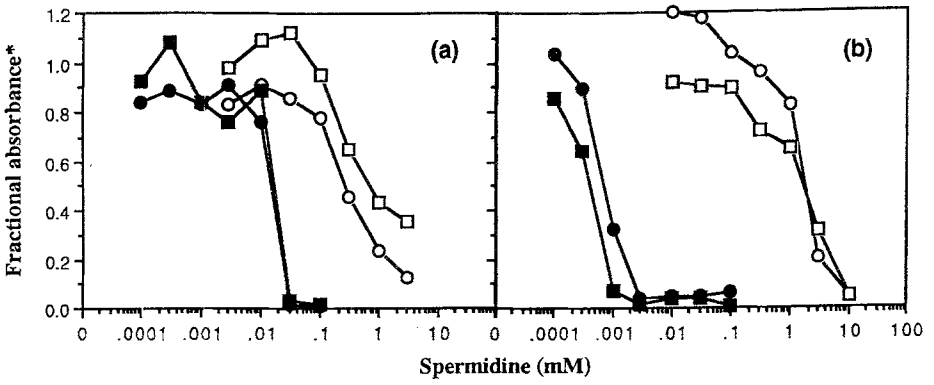


Fig. 1a, b The toxicity of spermidine in **a** EMT6/P and EMT6/AR1.0 and **b** H69/P and H69/LX4 cells in the presence of horse serum and bovine serum. ●, Parent, bovine serum; ○, parent, horse serum; ■, resistant, bovine serum; □, resistant, horse serum. The graphs represent typical data sets. Similar results were obtained in a number of independent experiments. * Fractional absorbance is defined by the mean optical density of the treated group divided by that of the control group

PBS. Then, 1 ml of distilled water was added to each well and the plates were left for 1 h at room temperature to allow cell lysis to occur. At the end of this time the contents of each well were pipetted several times and 500 µl was removed and placed in scintillant (Quicksafe, Zinsser Analytic). Radioactivity was measured the following day on a Beckman LS 5000CE liquid scintillation counter. The effect of deoxyspergualin on the ability of H69/P and H69/LX4 cells to accumulate [^{3}H]-daunorubicin was also determined. Cells inoculated at a concentration of 2×10^5/ml into 2-ml Eppendorf tubes in a volume of 1 ml were incubated at 37 °C. After 1 h deoxyspergualin at varying concentrations and cyclosporin A at 1 µ*M* were added to the appropriate tubes. The samples were then incubated for a further 30 min before the addition of [^{3}H]-daunorubicin to a final concentration of 0.1 µCi/ml. After this period the cells were rinsed three times with ice-cold PBS and finally lysed in 500 µl 0.1% sodium dodecyl sulphate (SDS). The radioactivity was measured as described for EMT6 cells.

Membrane vesicle preparation

Membrane vesicles were prepared from H69/P and H69/LX4 cells as previously described [1]. Exponentially grown cultures were washed twice in PBS containing the protease inhibitors, aprotinin (2 µg/ml), leupeptin (5 µg/ml) and pepstatin (0.08 µg/ml). The cells were then pelleted at 200 *g* for 5 min at 20 °C. Following this the cells were lysed in 1 m*M* TRIS (pH 7.4) containing protease inhibitors. The nuclei and unbroken cells were removed from the homogenate by centrifugation at 450 *g* for 10 min at 4 °C and the cell membranes were then separated from the resultant supernatant by centrifugation at 60,000 *g* for 1 h at 4 °C.

Photoaffinity labeling

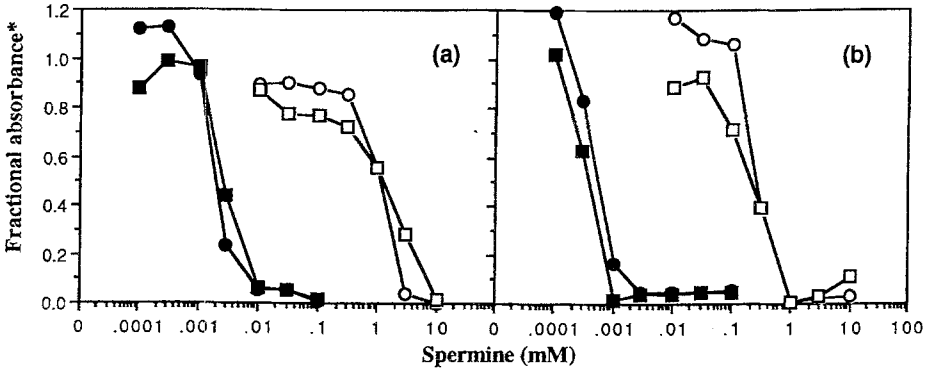
Membrane protein (50 µg) in a volume of 100 µl (1 m*M* TRIS-HCl, pH 7.4) was incubated for 1 h in the dark at room temperature with 0.03 µ*M* [^{3}H]-azidopine (Amersham; specific activity, 49 Ci/mmol) in the absence or presence of 5 µ*M* cyclosporin A or increasing concentrations of deoxyspergualin (1 – 100 µ*M*). After incubation, samples were irradiated in 96-well plates (Falcon) by placing the plates approximately 10 cm away from a UV lamp (wavelength, 360 nm) for 30 min on ice. For control purposes, one protein sample was exposed to [^{3}H]-azidopine but not to UV light. The labeled membranes were then run for 45 min at 200 V on a 7.5% SDS polyacrylamide gel. The gel was then fixed for 30 min in a solution of isopropanol: distilled water: acetic acid (25:65:10, by vol.) and enhanced in Amplify (Amersham) for a further 30 min. The enhanced gel was then dried under vacuum for 2 h and prepared for autoradiography. The fluorograms were exposed for 7 days.

Results

Toxicity of polyamines and deoxyspergualin

Both parent and resistant cell lines were more sensitive to the polyamines spermidine and spermine in the presence of

Fig. 2a, b The toxicity of spermine in **a** EMT6/P and EMT6/AR1.0 and **b** H69/P and H69/LX4 cells in the presence of horse serum and bovine serum. ●, Parent, bovine serum; ○, parent, horse serum; ■, resistant, bovine serum; □, resistant, horse serum. The graphs represent typical data sets. Similar results were obtained in a number of independent experiments. * Fractional absorbance is defined by the mean optical density of the treated group divided by that of the control group



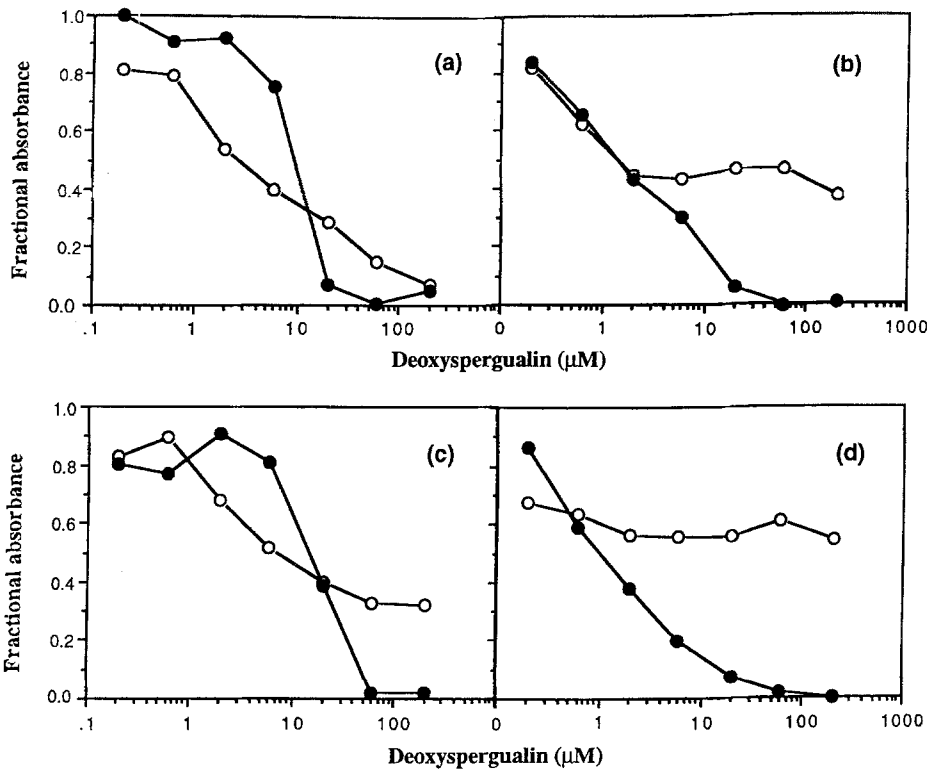


Fig. 3a–d The toxicity of deoxyspergualin in **a** EMT6/P, **b** H69/P, **c** EMT6/AR1.0 and **d** H69/LX4 cells in the presence of bovine serum (●) and horse serum (○). The graphs represent typical data sets. Similar results were obtained in a number of independent experiments. * Fractional absorbance is defined by the mean optical density of the treated group divided by that of the control group

bovine serum as compared with horse serum. Typical data sets are shown in Figs. 1–3. In the presence of bovine serum, EMT6/P, EMT6/AR1.0, H69/P and H69/LX4 cells were, respectively, 32-, 32-, 372- and 483-fold more sensitive to spermidine than in the presence of horse serum. The corresponding figures for spermine were 175-, 133-, 321- and 444-fold, respectively (Table 1). Interestingly, in the human cell lines H69/P and H69/LX4 the ratios of the doses of drug required to reduce fractional absorbance to 50% of the control value (IC_{50}) in bovine serum as compared with horse serum for spermine and spermidine were, respectively, 2- to 3- and 10- to 15-fold greater than in the mouse cell lines EMT6/P and EMT6/AR1.0. This may have been due to the differing levels of copper amine oxidase present in the two types of bovine serum (new-born calf serum in EMT6 cells and foetal calf serum in H69 cells) used to culture the different cell lines.

Deoxyspergualin is an analogue of spermidine. However, its relative toxic effects in the different serum types do not reflect those of the polyamines in the different serum types. When fractional absorbance was plotted against increasing drug concentration (Fig. 3), experiments carried out in the presence of bovine serum resulted in much steeper curves than those obtained in the presence of horse serum. The surviving fraction in horse serum-supplemented medium typically fell from 80% to 20% at between 1 and 100 μ*M* deoxyspergualin. In the bovine serum-supplemented medium a similar fall in the curves was produced by only a 2- to 5-fold dose increase. Similar results were obtained in a number of independent experiments.

EMT6/AR1.0 and H69/LX4 showed modest cross-resistance to deoxyspergualin, spermidine and spermine in both bovine serum and horse serum although this was only significant ($P < 0.05$; paired, two-tailed *t*-test) in EMT6/AR1.0 cells for deoxyspergualin in bovine serum and horse serum and for spermidine in horse serum alone (Table 2).

Table 1 Ratio of the IC_{50}[a] in bovine serum as compared with horse serum

	EMT6/P	EMT6/AR1.0	H69/P	H69/LX4
Spermine	200	133	320	443
	150	133	(68)	(33)
	$n = 2$	$n = 2$	$n = 4$	$n = 4$
Spermidine	31[b]	31	372	483
	(7)	(10)	(39)	(17)
	$n = 3$	$n = 3$	$n = 3$	$n = 3$

[a] The dose of drug required to reduce fractional absorbance to 50% of the control value

[b] Data shown are mean values (standard deviation) for *n* independent experiments. Where $n = 2$, individual values are shown

Effect of continuous exposure to deoxyspergualin on the colony-forming potential of EMT6/P and EMT6/AR1.0

To investigate further the differing response of EMT6/P and EMT6/AR1.0 cells to deoxyspergualin in the different serum types, we employed the clonogenic assay to examine the effect of deoxyspergualin (continuous exposure) on the number and size of colonies formed. The results obtained in the presence of bovine serum were similar to those produced in the MTT assay. The surviving fraction of colonies fell from 80% to 20% at between 2 and 20 μ*M* deoxyspergualin (Fig. 4). However, in the presence of horse serum the

Table 2 Resistance factors[a] for spermine, spermidine and deoxyspergualin (*BS* Bovine serum, *HS* horse serum)

	Spermine		Spermidine		Deoxyspergualin	
	BS	HS	BS	HS	BS	HS
EMT6/AR1.0 / EMT6/P	1.7[b] (0.2) $n = 4$	2.2 (1.2) $n = 4$	2.3 (1.5) $n = 4$	1.9* (0.5) $n = 4$	1.9* (0.7) $n = 6$	2.4* (0.4) $n = 6$
H69/LX4 / H69/P	1.3 (0.6) $n = 6$	1.7 (1.2) $n = 6$	1.2 (0.9) $n = 4$	1.4 (0.5) $n = 4$	1.2 (0.2) $n = 6$	1.8 (0.5) $n = 6$

* $P < 0.05$; value is significanty different from that of the control (paired, two-tailed *t*-test)

[a] Resistance factor $= \frac{IC_{50} \text{ of drug in resistant cells}}{IC_{50} \text{ of drug in parent cells}}$

[b] Data are mean values (standard deviation) for *n* independent experiments

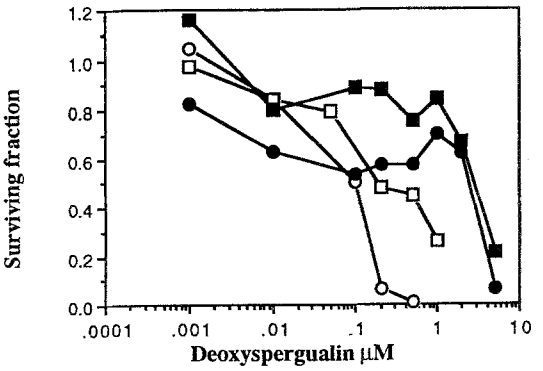


Fig. 4 Effect of deoxyspergualin on the colony-forming ability of EMT6/P and EMT6/AR1.0 cells in the presence of either horse serum [EMT6/P (○), EMT6/AR1.0 (□)] or bovine serum [EMT6/P (●), EMT6/AR1.0 (■)]. The graph represents typical data sets. Similar results were obtained in a number of independent experiments

curve dropped more steeply than the curve produced in the MTT assay. This is likely to have been due to the observation that in horse serum, as the deoxyspergualin concentration increased, colonies reduced in size (i.e. number of cells per colony) rather than in number. Only those colonies with 50 or more cells were included in the count. At higher doses of deoxyspergualin (>0.1 μ*M*) there were many colonies containing fewer than 50 cells, which were therefore not included in the count. This accounts for the steeper curve obtained in this assay as compared with the MTT assay, which is contributed to by all viable cells without an arbitrary cut-off point. These data indicate that deoxyspergualin is cytostatic to EMT6/P and EMT6/AR1.0 cells in the presence of horse serum and cytotoxic in the presence of new-born calf serum.

Effect of deoxyspergualin on the accumulation of [^{3}H]-daunorubicin in EMT6/AR1.0 and H69/LX4 cells

There was no difference in the accumulation of [^{3}H]-daunorubicin between experiments carried out using medium supplemented with either 10% bovine serum or 10% horse serum in either the parent or resistant cell line. Figure 5 shows that deoxyspergualin at concentrations of up to 200 μ*M* did not reverse the accumulation deficit in EMT6/AR1.0 or H69/LX4 cells. In comparison, cyclosporin A (1 μ*M*) had an accumulation ratio of 17.6 in EMT6/AR1.0 cells (where the accumulation ratio is defined by the drug uptake in the presence of modifier divided by the drug uptake in the absence of modifier) and restored [^{3}H]-daunorubicin accumulation to levels comparable with those seen in the parent line. The differential accumulation observed between parent and resistant cells was much lower in the human cell line than in the mouse cell line. Cyclosporin A (1.0 μ*M*) did not reverse the accumulation deficit in LX4 cells to the same extent as it did in EMT6/AR1.0 cells. However, cyclosporin A (5.0 μ*M*) restored [^{3}H]-daunorubicin accumulation to levels higher than that of the parent line.

Effect of deoxyspergualin on the sensitivity of EMT6/AR1.0 and H69/LX4 cells to doxorubicin

There was no significant difference between the IC_{50} values obtained for of EMT6/AR1.0 in the presence or absence of deoxyspergualin in either serum type. However, deoxysper-

Fig. 5a The effect of cyclosporin A (*CsA*) and deoxyspergualin (*DSG*) on the cellular uptake of [^{3}H]-daunorubicin (*DNR*) in EMT6/P (*P*) and EMT6/AR1.0 (*R*) cells. The graph represents typical data sets. Similar results were obtained in a number of independent experiments. **b** The effect of cyclosporin A (*CsA*) and deoxyspergualin (*DSG*) on the cellular uptake of [^{3}H]-daunorubicin (*DNR*) in H69/P (*P*) and H69/LX4 (*R*) cells. The graph represents typical data sets. Similar results were obtained in a number of independent experiments

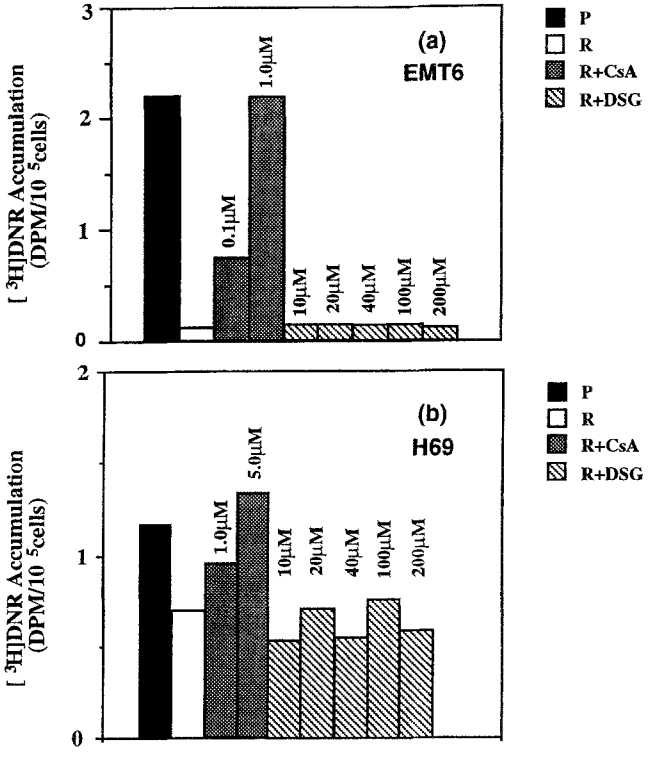


Table 3 Effect of deoxyspergualin on the IC_{50} of doxorubicin in EMT6/AR1.0 and H69/LX4 cells (*BS* Bovine serum, *HS* horse serum)

	EMT6/AR1.0 (IC_{50}, μM)		H69/LX4 (IC_{50}, μM)	
	BS	HS	BS	HS
Control	7.9[a] (2.6)	4.0 (2.4)	1.1 (0.5)	1.3 (0.5)
Deoxyspergualin (2 μM)	8.0 (2.6)	7.5 (3.7)	1.5 (0.8)	1.8 (0.4)
Deoxyspergualin (4 μM)	6.2 (1.0)	7.8 (4.5)	1.7 (0.9)	1.6* (0.5)

* $P < 0.05$; value is significantly different from that of the control (paired, two-tailed *t*-test)

[a] Data values are means (standard deviation) from at least 3 independent experiments

gualin (4 μM) caused a modest but significant decrease in the IC_{50} of doxorubicin in H69/LX4 cells in the presence of horse serum ($P < 0.05$; paired, two-tailed *t*-test). In the presence of bovine serum there was no effect (Table 3).

Inhibition of [^{3}H]-azidopine covalent binding to P-glycoprotein in membranes prepared from H69/LX4 cells

The potential of deoxyspergualin to inhibit the covalent binding of [^{3}H]-azidopine to P-glycoprotein was studied using the photoaffinity-labeling technique. Figure 6 shows that the covalent binding of [^{3}H]-azidopine was seen only after exposure to UV irradiation. With UV exposure there was a clear band at a molecular weight of 170 kDa in H69/LX4 cells but not in H69/P cells, indicating the presence of [^{3}H]-azidopine binding to P-glycoprotein. Covalent binding of [^{3}H]-azidopine to P-glycoprotein occurred only in the resistant cell line after UV irradiation. Deoxyspergualin at concentrations ranging between 2 and 40 μM did not inhibit the covalent binding of [^{3}H]-azidopine to P-glycoprotein. In contrast, however, clear inhibition was produced by cyclosporin A (1 μM).

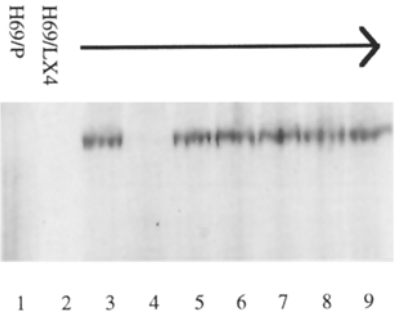


Fig. 6 Effect of deoxyspergualin (DSG) on the covalent photoincorporation of [^{3}H]-azidopine to P-glycoprotein in H69/P membranes (*lane 1*) and H69/LX4 membranes (*lanes 2–9*). (*Lane 2* no UV exposure, *lane 3* no modifier, *lane 4* + CsA at 5 μM, *lanes 5, 6, 7, 8 and 9*, + DSG at 2, 4, 10, 20 and 40 μM, respectively) The figure represents typical data sets. Similar results were obtained in a number of independent experiments

Discussion

Copper amine oxidase contained in serum is thought to be responsible for the cytotoxicity of polyamines and deoxyspergualin in vitro [15]. Our studies support the theory that aminoaldehydes and hydrogen peroxide produced by the oxidative deamination of polyamines by copper amine oxidases are responsible for the cytotoxicity of these agents in vitro in the presence of bovine serum. However, the results we obtained using horse serum, which is low in copper amine oxidase content, indicate that this may not be the only mechanism of toxicity. Spergualin has potent antitumour activity in mouse neoplasms [16]. Mouse serum is poor in copper amine oxidase [15]; therefore, the antitumour effect of deoxyspergualin is unlikely to be due to its oxidation in murine blood. Hence, the mechanism of cytotoxicity in this system may involve factors other than oxidative deamination by copper amine oxidase. The antitumour activity of spergualin is markedly reduced in immunodeficient mice [8]. It may therefore be possible that in vivo in the presence of serum types that are low in copper amine oxidase, e.g. mouse and human serum, the anti-tumour activity of deoxyspergualin may be due to activation of the immune system.

Kuramochi et al. [7] have also shown that the antiproliferative action of deoxyspergualin is different from that induced by copper amine oxidase. This group demonstrated that aminoguanidine, a potent inhibitor of copper amine oxidase activity, did not decrease the growth-inhibitory activity of deoxyspergualin in the presence of human serum, which is low in copper amine oxidase activity. They also showed that the survival curves produced by exposure of cells to deoxyspergualin in the presence of human serum were shallow and therefore concluded that this may be due to the mechanism of action of deoxyspergualin in the presence of human serum being cytostatic rather than cytocidal. The survival curves we produced by plotting the surviving fraction, determined by measuring fractional absorbance in the MTT assay, against the $\log_{10}$ value (concentration of drug) were also shallow in the presence of horse serum. Our results therefore support those of Kuramochi et al. [7], and it appears that in the absence of copper amine oxidase the mechanism of action of deoxyspergualin may indeed be cytostatic.

Nishikawa et al. [12] showed that deoxyspergualin arrested cells in the G_0/G_1 phase and reduced the cycling cell population. Conversion from the G_0 to the G_1 phase and progression to the S phase are two critical steps for cell proliferation. Our results show that in the absence of the copper amine oxidase present in bovine serum, deoxyspergualin appears to exert a cytostatic effect on the cell population, reflecting the drug's effect on the cell cycle.

Cyclosporin A and FK506 are natural products possessing potent immunosuppressive properties as well as the capability of selectively restoring sensitivity to cells with acquired multidrug resistance. By studying the uptake of

radiolabeled daunorubicin we could show that unlike cyclosporin A and FK506, deoxyspergualin did not alter the drug accumulation deficit present in the cell line EMT6/AR1.0.

The use of the photo-active tritiated arylazide azidopine enabled us to demonstrate that resistance modifiers such as cyclosporin A are capable of inhibiting the covalent binding of [^{3}H]-azidopine to P-glycoprotein. The results obtained in our photoaffinity-labeling experiments show that deoxyspergualin does not inhibit the covalent binding of azidopine to P-glycoprotein, suggesting that it is not a substrate for the protein. This is supported by the observations that deoxyspergualin does not reverse the accumulation deficit in EMT6/AR1.0 or H69/LX4 cells and that it is not capable of restoring the sensitivity to doxorubicin of the drug-resistant cell lines EMT6/AR1.0 or H69/LX4 to the levels of the parent cells, indicating that deoxyspergualin does not modify classical multidrug resistance.

The Pgp-hyperexpressing, human multidrug-resistant variant H69/LX4 shows no cross-resistance to deoxyspergualin. The mouse EMT6/AR1.0 cells, however, show modest but significant cross-resistance to the drug. Deoxyspergualin is not a substrate for human P-glycoprotein, as we demonstrated using photoaffinity labeling, and therefore is not removed from the human cells by the active transport drug-efflux pump. It is possible that deoxyspergualin has a low but significant affinity for mouse P-glycoprotein; however, the cross-resistance observed in the mouse line may be due to an alternative mechanism other than increased efflux by P-glycoprotein.

Cyclosporin A binds to a cytosolic protein, cyclophilin. FK506 also binds to an intracellular binding protein. Both these proteins have been shown to possess peptidyl-propyl cis-trans isomerase activity. If cyclosporin A and FK506 exert their resistance-modifying activity, at least in part, by the inhibition of peptidyl-propyl cis-trans isomerase activity, then this may account for the lack of activity of deoxyspergualin, which does not inhibit this enzyme.

Our data suggest that unlike cyclosporin A and FK506, deoxyspergualin is not a good candidate for development as a resistance modifier. However, its maintenance of activity in classical multidrug-resistant cells and its potent in vivo anti-tumour activity make deoxyspergualin a promising agent for further investigations into its potential clinical use as an anti-tumour agent.

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