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Circumvention of Pleiotropic Drug Resistance in Subcutaneous Tumours *in vivo* with Verapamil and Clomipramine

Stephen Merry, Thomas G. Hamilton, Patricia Flanigan, R. Ian Freshney and Stanley B. Kaye

The development of pleiotropic drug resistance (PDR) *in vivo* in solid tumour models suggests that a similar process may occur in the clinic. A subline of the Ridgway osteogenic sarcoma (ROS)—a murine subcutaneously-growing solid tumour—with moderate resistance (1.5 fold) to actinomycin D was selected by repeated suboptimal treatment with this drug *in vivo*. This subline (ROS/ADX/G2) showed cross-resistance to vincristine (3.5 fold) and etoposide (over 5.1 fold) but not to doxorubicin. The resistance could in all cases be partly or completely overcome by treatment with non-cytotoxic doses of verapamil or clomipramine. Resistance to actinomycin in this model was associated with lower (up to 3.2 fold) drug accumulation into tumours which could be increased (up to 2.8 fold) by treatment with 25 μg/g verapamil. These data support clinical trials of the use of membrane-active agents to overcome PDR.
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INTRODUCTION

WHEN RESISTANCE to actinomycin D develops *in vitro*, it is commonly observed that the cell line becomes cross-resistant to anthracyclines, vinca alkaloids and epipodophyllotoxins [1]. This finding, termed pleiotropic drug resistance (PDR), has two important characteristics. Firstly, it is associated with reduced intracellular drug levels, and secondly, PDR can be overcome at least *in vitro* by membrane-active agents.

There have been few studies of PDR in solid tumours *in vivo*. We have developed an actinomycin D resistant subline of a murine subcutaneous tumour, the Ridgway osteogenic sarcoma (ROS). In this model we have investigated (i) the pattern of cross-resistance to doxorubicin, vincristine and etoposide (ii) the effects of the calcium antagonist verapamil and the antidepressant clomipramine on drug resistance, and (iii) the effects of verapamil on actinomycin D accumulation and retention. These studies parallel and extend those of Kaye and Bowden [2] who used a different resistant subline of ROS.

MATERIALS AND METHODS

Drugs

Actinomycin D was from Merck Sharp & Dohme, clomipramine from Geigy Pharmaceuticals, doxorubicin from Farmitalia Carlo Erba, verapamil from Abbott Laboratories, vincristine from Eli Lilly and etoposide from Bristol Myers. The drugs were solubilised according to the manufacturers' instructions for clinical use and stored (generally for no longer than a month) as frozen aliquots at −20°C. Clomipramine was further diluted in sterile distilled water immediately before injection; the others in sterile phosphate buffered saline. [^{3}H]Actinomycin D (Amersham International) was stored at −20°C.

Correspondence to S. Merry, Imperial Laboratories (Europe) Ltd, West Portway, Andover, Hampshire SP10 3LF, U.K.
The authors are at the CRC Department of Medical Oncology of the University of Glasgow, Garscube Estate, Bearsden Road, Glasgow G61 1BD, U.K.
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Mice

AKR mice aged 6–18 weeks were used with approximately equal numbers of males and females in each treatment group. The mice were bred within our department. All animals were fed standard laboratory diet and water *ad libitum* and conditions of heating and lighting were constant.

Tumour models

ROS was a gift from the late Dr F.M. Schabel, Jr (Southern Research Institute, Birmingham, Alabama) and was maintained in Charing Cross Hospital Medical School animal unit before transfer to Glasgow. The tumour line was maintained by subcutaneous passage of 20 mm^3 fragments in the right flank of female mice.

A resistant tumour line (ROS/ADX/G2) was developed from ROS by repeated suboptimal treatment (0.3 μg/g intraperitoneally) with actinomycin D of tumour-bearing female animals followed by passage of the fastest growing tumours. The experiments described here were done after at least seven transplant generations when an apprarently stable level of resistance was obtained. ROS and ROS/ADX/G2 have an almost identical morphology [3].

Drug sensitivity

Tumour size was estimated according to the formula $(a \times b^2)/2$ where a is the largest superficial diameter and b is the perpendicular height. When measurements are made in cm this formula has been equated with tumour weight in g [4]. In preliminary experiments with tumours ranging in size from 0.3 to 8.4 g measurements of both tumour height and length were reproducible within a range of 0.2 cm. To assess drug sensitivity,

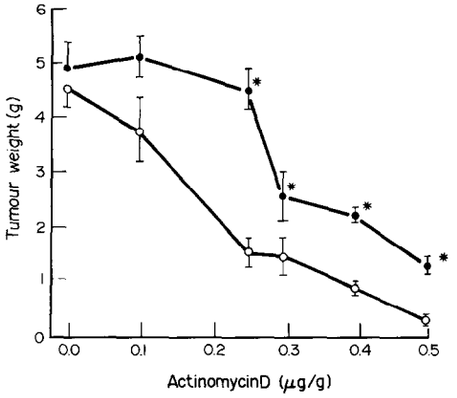

Fig. 1. Dose-response curves showing sensitivity of ROS (○—○) and ROS/ADX/G2 (●—●) to actinomycin. Mean (S.E.) n = 5–10. *$P < 0.05$, t test.

groups of 5–10 mice bearing sensitive or resistant tumours of 1–1.5 g were either untreated or treated with saline or cytotoxic drugs—(μg/g) 0.125–0.5 actinomycin D, 2.5–15 doxorubicin, 0.25–2.0 vincristine or 5–40 etoposide—and/or 25 μg/g verapamil or 10 μg/g clomipramine. To protect against observer bias treatment groups were randomised between cages. In some cases the dose of verapamil or clomipramine was repeated after 24 h. All drugs were given intraperitoneally in 0.01–0.02 ml/g. Tumour growth was measured at 10 days (the point of maximum tumour regression).

Drug accumulation

Groups of 5–10 mice bearing ROS or ROS/ADX/G2 tumours of 2–4 g were treated with [^{3}H] actinomycin D (specific activity 3.1 TBq/mol) at 0.6 μg/g (about 37 kBq per mouse). At 3, 24, 48, 72 and 168 h groups of mice were killed. Their tumours were excised, dissected free of necrotic material, weighed and stored at −20°C for up to a week. The tumours were thawed at room temperature, digested in three volumes of 33% KOH overnight and 0.2 ml aliquots were neutralised (1 ml 1.5 mol/l HCl) and counted after the addition of 10 ml Ecoscint (National Diagnostics Inc). This procedure was similar to that used previously by Kaye *et al.* [4].

RESULTS

Figure 1 shows the effect of increasing doses of actinomycin D on the growth of ROS and ROS/ADX/G2 tumours. The ID_{50} for ROS/ADX/G2 was 52% greater than that for ROS (0.32 and 0.21 μg/g, respectively). Dose-response curves were plotted for doxorubicin, vincristine and etoposide (data not shown), and statistically significant differences in tumour weights between ROS and ROS/ADX/G2 were found at 0.5 and 1.0 μg/g vincristine and 10, 20 and 40 μg/g etoposide. The shape of these curves was similar to those obtained with actinomycin and the results are summarised in Table 1. For ROS/ADX/G2 it was not possible to determine the ID_{50} for etoposide since doses over 40 μg/g produced significant mortality. ROS/ADX/G2 was cross-resistant to vincristine and etoposide, and for these drugs the degree of resistance was greater than that to actinomycin D, the compound used to induce resistance. The degree of cross-resistance to doxorubicin was, however, marginal.

Table 1. Sensitivity of ROS and ROS/ADX/G2 to cytotoxic drugs

Drug	ID_{50} (μg/g) ROS	ID_{50} (μg/g) ROS/ADX/G2	Ratio
Actinomycin D	0.21	0.32	1.52
Etoposide	7.80	>40.00	>5.12
Vincristine	0.35	1.24	3.54
Doxorubicin	6.60	7.50	1.14

Table 2. Sensitivity of ROS and ROS/ADX/G2 to verapamil and clomipramine

Treatment (mg/g)	Tumour weight at 10 days (g) ROS	Tumour weight at 10 days (g) ROS/ADX/G2
None	4.9 (0.5)*	4.6 (0.5)
Verapamil		
1 × 25	4.3 (0.6)	6.3 (0.4)
2 × 25	5.5 (0.5)	4.9 (0.4)
Clomipramine		
1 × 10	5.0 (0.4)	5.0 (0.3)
2 × 10	5.2 (0.3)	5.2 (0.3)

*Mean (S.E.). n = 5–10.

Table 2 shows the effect of treatment with maximum tolerated doses of clomipramine and verapamil on the growth of ROS and ROS/ADX/G2. In no case was a significant inhibition of tumour growth observed. These non-cytotoxic doses of clomipramine and verapamil were combined with doses of cytotoxic drugs that produced moderate reductions in tumour size (48% for actinomycin D, 15% for vincristine and 34% for etoposide) when administered alone to ROS/ADX/G2. The experimental data for actinomycin D are shown in Fig. 2. For ROS/ADX/G2 the addition of one or two doses of 25 μg/g verapamil or two doses of 10 μg/g clomipramine to 0.3 μg/g actinomycin D produced a significant reduction in tumour size compared with actinomycin alone. These data are summarised together with the results for

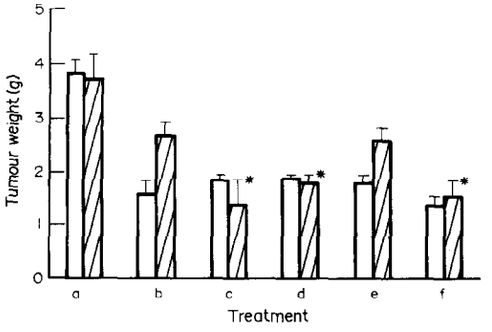

Fig. 2. Effect of verapamil and clomipramine on sensitivity of ROS (□) and ROS/ADX/G2 (▨) to 0.3 μg/g actinomycin D. Mean (S.E.), n = 5–10. (a) = control (untreated), (b) = actinomycin D alone, (c) = plus 25 μg/g verapamil, (d) = plus two doses of 25 μg/g verapamil, (e) = plus 10 μg/g clomipramine and (f) = plus two doses of 10 μg/g clomipramine. *$P < 0.05$, t test.

Table 3. Effect of verapamil and clomipramine on ROS/ADX/G2 resistance to actinomycin, etoposide and vincristine

Cytotoxic treatment (day 0)	Additional treatment (day 0 or days 0 and 1)				
		Verapamil		Clomipramine	
	PBS	Once	Twice	Once	Twice
0.3 μg/g actinomycin D	1.74*†	0.77	0.96	1.51	1.20
40 μg/g etoposide	1.76†	1.51	0.91	1.73†	0.76
0.5 μg/g vincristine	1.83†	1.12	0.96	1.19	1.30

*Mean weight (ROS/ADX/G2)/(ROS) at 10 days. Ratio over 1 indicates relative resistance, 1 or under indicates lack of resistance.
†Significant ($P < 0.05$, t test) difference between weights of ROS and ROS/ADX/G2 tumours.

vincristine and etoposide in Table 3, where the relative resistance of ROS/ADX/G2 is expressed as the ratio of tumour weights at 10 days. Two treatments with 10 μg/g clomipramine completely overcame resistance to actinomycin D and vincristine, and, in the case of vincristine, a single treatment with clomipramine could partly overcome resistance. Two treatments with 25 μg/g verapamil completely overcame resistance to actinomycin D, vincristine and verapamil, and, for actinomycin D and vincristine, a single treatment was sufficient to overcome resistance.

Figure 3 shows the levels of radioactivity in tumour homogenates obtained from animals treated with labelled actinomycin D. This dose of actinomycin D and the use of animals bearing large tumours meant that tumour size remained approximately constant (for both ROS and ROS/ADX/G2) throughout the experiment. The data show that in the absence of verapamil peak levels of radioactivity in ROS are 3 fold higher than those in ROS/ADX/G2 and that the total area under the curve of ROS is 3.5 fold that of ROS/ADX/G2. Treatment with 25 μg/g verapamil reduces the difference in peak heights to 1.5 fold and the difference in area under the curve to 1.1 fold.

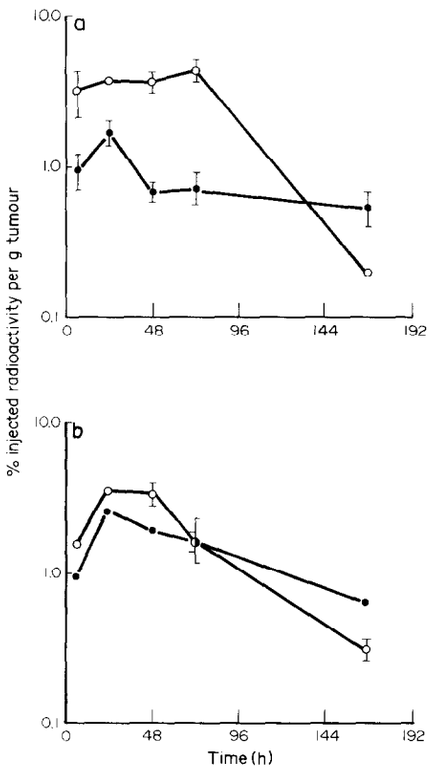

Fig. 3. Radioactivity in ROS (○—○) and ROS/ADX/G2 (●—●) tumours after treatment with 0.6 μg/g [^{3}H]actinomycin D (a) alone or (b) plus 25 μg/g verapamil. S.E. omitted if range smaller than size of symbol. n = 5–10.

DISCUSSION

The clinical relevance of PDR is yet to be established although there is some evidence that it may contribute to treatment failure in some patients. A 180 kD membrane glycoprotein (the *P*-glycoprotein) has been found in many rodent and human cell lines exhibiting PDR [5] and, with monoclonal antibodies, this glycoprotein has been demonstrated in biopsy specimens of human leukaemia [6], ovarian cancer [7] and sarcomas [8]. Fojo *et al.* [9] have also shown the presence of mRNA coding for the *P*-glycoprotein in several human tumours, including those derived from adrenal gland and colon.

The drug resistant ROS/ADX/G2 murine solid tumour line was developed by repeated suboptimal treatment of mice bearing ROS tumours—i.e. in a manner analogous to that which might occur in the clinic. Resistance was developed by treatment with actinomycin D and cross-resistance occurred to vincristine and etoposide but not to doxorubicin. Two previous reports have documented the development of resistance to actinomycin D *in vivo* [2, 10].

Verapamil [11–14] and clomipramine [14] overcome PDR *in vitro*. These agents administered at doses that do not inhibit tumour growth overcame resistance in ROS/ADX/G2, suggesting that the mechanism of resistance in this model is similar to that occurring in other models in which resistance was derived *in vitro*. PDR is associated with decreased intracellular drug levels *in vitro*, which are increased in the presence of verapamil. In our studies of accumulation of actinomycin D into ROS and ROS/ADX/G2, verapamil had little effect on drug accumulation into ROS tumours (which accumulated up to 3 fold more actinomycin D than ROS/ADX/G2), while causing a two fold increase in drug accumulation into ROS/ADX/G2. Kaye *et al.* [4] showed that similar levels of drug were initially present in their resistant and sensitive ROS tumour lines, but drug was cleared more rapidly from resistant tumours. Our data for the parental ROS tumour line are similar to those of Kaye *et al.* [11] and Schwartz *et al.* [15], but our data for ROS/ADX/G2 differ from those of Kaye *et al.* [4] for their resistant tumour line in that we found lower levels of drug in ROS/ADX/G2 at 3 h. The difference between the two tumour models could be either intrinsic (as indicated by their differing patterns of cross-resistance) or result from different routes (intraperitoneal vs. intravenous) of delivery of actinomycin D. There has been only one study of the effect of verapamil on drug accumulation into solid tumours *in vivo*. Formelli *et al.* [16] reported that verapamil did not alter doxorubicin uptake into either sensitive or resistant sublines of B16 melanoma.

There have been a few reports that verapamil increases sensitivity to cytotoxic drugs in various tumour models *in vivo*. Robinson *et al.* [17] and Formelli *et al.* [16] reported increases in sensitivity to melphalan and doxorubicin, respectively, in murine tumour lines. Mattern *et al.* [18] and Merry *et al.*

(ref. 19 and unpublished data) found increased sensitivity to vincristine and etoposide, respectively, in human lung cancer xenografts. Ikeda *et al.* [20] reported increased sensitivity to cisplatin in a human neuroblastoma xenograft. The results for melphalan and cisplatin are particularly interesting since these drugs are not normally associated with PDR [1].

There have been a limited number of clinical studies of the use of verapamil to overcome cytotoxic drug resistance. In phase I/II studies Presant *et al.* [21] reported a partial response to doxorubicin plus verapamil in a patient with doxorubicin-resistant pancreatic carcinoma and Cantwell *et al.* [22] reported responses in patients with non-small cell lung cancer to the combination of vindescine with verapamil. Ozols *et al.* [23], however, found that verapamil did not enhance the effectiveness of doxorubicin in ovarian cancer. Phase III preliminary results [24] showed that verapamil may enhance the effectiveness of combination chemotherapy including doxorubicin, vincristine and etoposide in small cell lung cancer.

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