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The comparative disposition of the pyrolloquinone GR63178A and its 9-hydroxy metabolite GR54374X in sensitive and resistant mouse colon adenocarcinoma

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Abstract. The novel anticancer compound GR63178A is being evaluated in the clinic, having demonstrated activity against a wide range of experimental tumour systems in animals without significant toxic side-effects being apparent. In this work, we have demonstrated significant antitumour action of this compound against one murine colon cancer model (colon 38 tumour in BDF-1 mice, specific growth delay = 1.2) when given at 10 mg/kg over 21 consecutive days and in contrast shown minimal sensitivity of another similar murine colon adenocarcinoma, MAC 26, in NMRI mice with the same dose regime. We investigated the disposition of both the parent drug and the 9-OH metabolite (GR54374X) in plasma, tissues and tumours, using solid phase extraction followed by reversed-phase high performance liquid chromatography. Although plasma clearance profiles of GR63178A were similar, significant differences were seen in the disposition of the drug to major organs in two mouse strains. Noteably, the liver and kidneys of the sensitive model had higher levels of parent drug and 9-OH metabolite at both 30 min and 4 h post-injection. However, this was not apparent in the tumours themselves, and the levels of 9-OH metabolite were lower in the plasma and higher in the urine of the sensitive mice, indicating possible rapid renal clearance of this compound. Neither GR63178A nor GR54374X proved cytotoxic in in vitro experiments. The data presented here have revealed considerable variation in drug handling by these two mouse strains, but this did not produce different levels of either parent drug or GR54374X in the tumours, which are the presumed targets, suggesting that differences in disposition are probably not responsible for the different sensitivities of the two tumours. Other possible explanations include the production of a hitherto undetected ultimate cytotoxic metabolite in the sensitive, but not in the resistant, mouse/tumour combination, or differences in inherent tumour sensitivity, or in host-mediated effects. These possibilities are discussed.

Introduction

Mitoquidone (NSC 382057D, GR30921; Fig. 1) is a pentacyclic pyrolloquinone, the lead compound of a series of anticancer agents with an unusual spectrum of activity and minimal apparent toxicity in animals [9]. Mitoquidone was used in phase I clinical trials [15] which were halted before the maximum tolerated dose was reached or a tumour response was recorded, due to poor water solubility and consequent problems with formulation. GR63178A (Fig. 1) is an analogue of mitoquidone with a similar spectrum of activity, but possessing enhanced water solubility. As with mitoquidone, this compound has a high therapeutic index in animals [10]. In clinical phase I studies, observed side-effects included nausea, vomiting, phlebitis and pain at the tumour site; the latter effect is unusual for a conventional cytotoxic drug [8, 16].

In animals the drug is required to be given chronically, up to 35 consecutive days of dosing, for full expression of activity. In such regimes, GR63178A has been shown to inhibit the growth of murine tumours including sarcoma 180, MAC 30T adenocarcinoma and rat D23 hepatoma, although no activity against MAC 15 was apparent. It is also active against human tumour xenografts in nude mice, including mammary, lung, ovarian and colon (HT29) tumours [10]. However, only two of ten ovarian tumour lines grown in NMRI Cpb nude mice showed even moderate sensitivity to GR63178A, although sensitivity to the lead compound mitoquidone was apparent in four out of four tumour lines tested [2]. Interestingly, activity against HT29 colon tumour xenografts is not mirrored by activity against the same tumour as a cell line in vitro. Indeed, no

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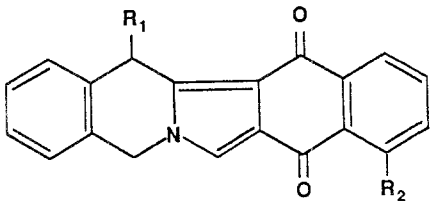


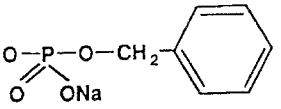
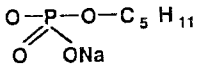
Name	R_1	R_2
Mitoquidone	H	H
GR 63178A	CH_3	
GR 54374X	CH_3	OH
GR 70440A	CH_3	

Fig. 1. Structures of GR63 178A, a major 9-hydroxy metabolite GR54 374X and GR70 440A (internal standard)

activity is reported against any in vitro system, or against murine L1210 and P388 leukaemias in vivo [10].

Differences in the sensitivity of tumour models to GR63 178A may be due to variables which include metabolism, disposition, modulation of the immune system, tumour blood flow or subcellular processes which predispose certain tumour lines to sensitivity (for example repair of DNA-drug adducts). We have compared the sensitivity of two mouse models bearing murine colon adenocarcinomas: hairy NMRI mice with MAC 26 tumour, and BDF-1 mice with colon 38 (C38). We have subsequently investigated the disposition of GR63 178A and its metabolite, the 9-OH derivative GR54 374X in these two murine models.

Materials and methods

Chemicals. Pure GR63 178A (sodium salt), GR54 374X and GR70 440A (sodium salt, internal standard) were kindly supplied by the Infection and Oncology Department of the Medical Division of Glaxo Group Research Ltd, Greenford, Middlesex, England. Standard solutions were prepared in a darkroom to avoid photodegradation [11]. Acetonitrile, methanol and ethanol were from Rathburn Chemicals, Walkerburn, Scotland. Ammonium acetate and acetic acid were of HPLC grade and purchased from Fisons, Loughborough, England. *N,N*-Dimethylacetamide (DMA; Analar grade) was from British Drug Houses, Poole, England. All other reagents were of Analar grade or equivalent; water was double distilled and deionised in a quartz still on site.

Tumour growth. Male NMRI or BDF-1 mice were kept in laboratory cages and fed on SDS CRM-X rodent diet (Special Diet Services, Annan, Scotland) and water ad libitum. Mice used for analysis of urine were kept in metabolic cages with urine and faeces collection traps insulated against light with aluminium foil.

Mice were inoculated by trochar subcutaneously in one flank with approximately 8 mm^3 pieces of either colon 38 tumour (in BDF-1 mice; tumour supplied by L'Institut Jules Bordet, Brussels, Belgium) or MAC 26 tumour (in NMRI mice; tumour was supplied by Dr. John Double, Clinical Oncology Unit, University of Bradford, UK). Both tumours are moderately to well differentiated, non-mucinous murine colon adenocarcinomas with a tubular microscopic morphology, induced originally by 1,2 dimethylhydrazine dichloride insult to the host mouse [3, 5]. Inoculations were performed on mice aged 12–14 weeks; BDF-1 mice weighed 19.8–25.4 g (mean 22.5 g); NMRI mice weighed 20–23.2 g (mean 21.4 g). Efficiency of tumour inoculation (tumour take-rate) was measured and numbers of tumours established were expressed as a percentage of total inoculations. After 1 day post-inoculation, BDF-1 or NMRI mice were treated by i.p. injection with 10 mg/kg GR63 178A or an equivalent volume of injection vehicle only (controls). This regime was repeated daily for 21 days. The growth of the tumours was monitored on alternate days for the duration of the drug treatment and for up to 2 weeks after dosing had finished. Tumours were measured with calipers along the length (i.e. longest diameter) and width (perpendicular to the longest diameter). Volume was calculated as:

$$\text{Volume} = 0.5 \times \text{length} \times \text{width}$$

Tumour growth was expressed as volume (cm^3) and specific growth delay (SGD) was calculated as:

$$\text{SGD} = \frac{\text{Td(control)} - \text{Td(treated)}}{\text{Td(treated)}},$$

where Td(control) and Td(treated) are the tumour doubling times for control and treated mice respectively. Using SGD measurements enables direct comparisons between tumour models with different inherent growth kinetics (after [14]).

Drug metabolism and disposition. Mice were prepared by trochar injection of tumour pieces as described above. Tumours were allowed to reach a size of 0.4 cm^3 (after approximately 14 days for colon 38 and 21 days for MAC 26). Mice were then treated with single injections of GR63 178A (i.p., 10 mg/kg). Animals were killed by etherisation at appropriate time points after administration of GR63 178A, and blood was removed from the dorsal aorta into heparinised syringes and centrifuged immediately to separate plasma, which was aspirated and stored in cryotubes in liquid nitrogen until required for analysis. Solid tissues were removed, washed in ice-cold phosphate-buffered saline and stored in cryotubes in liquid nitrogen until required for analysis.

Tissue and plasma sample preparation and analysis. Wherever possible, all plasma, tissue and extract manipulations were carried out in a darkroom equipped with a red safelight, to avoid photodegradation of the parent drug [11]. Plasma samples were defrosted and a known amount of GR70 440A internal standard was added. After thorough mixing, the samples were loaded onto Bond-Elut C2 sample preparation columns as previously described [6]. After washing the column with water and eluting parent drug and metabolites in methanol, the eluates were dried on a Uniscience Univap centrifugal evaporator and reconstituted in mo-

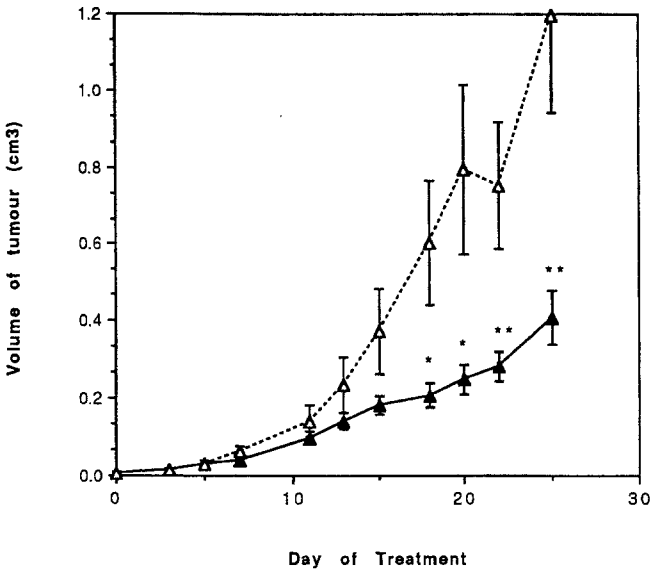


Fig. 2. Growth curve of colon 38 tumour as subcutaneous implant in BDF-1 mice; untreated control (*broken line, open symbols*) or with 10 mg/kg GR63 178A on days 1–21 (*solid line, closed symbols*). *Points* are means ± SEM of at least five mice with tumours. Significant differences from control tumour volumes: * $P < 0.05$; ** $P < 0.01$

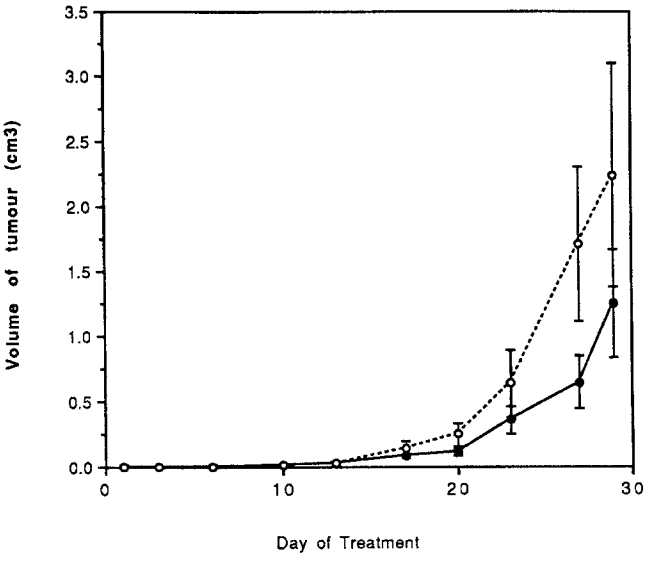


Fig. 3. Growth curve of MAC 26 tumour as subcutaneous implant in NMRI mice; untreated control (*broken line, open symbols*) or with 10 mg/kg GR63 178A on days 1–21 (*solid line, closed symbols*). *Points* are means ± SEM of at least five mice with tumours. Differences in tumour volumes did not reach statistical significance

bile phase prior to analysis by HPLC. Solid tissues were defrosted and homogenised on ice (20% w/v) in cold phosphate-buffered saline (pH 7.1), to which a known amount of GR70 440A internal standard had been added. The total homogenate was then centrifuged for 10 min to remove gross cell debris, and the resultant supernatant was loaded onto Bond-Elut C2 sample preparation columns and treated similarly to plasma samples. Mouse urine samples were spiked with known amounts of GR70 440A internal standard and then loaded directly onto the HPLC for analysis without further preparation.

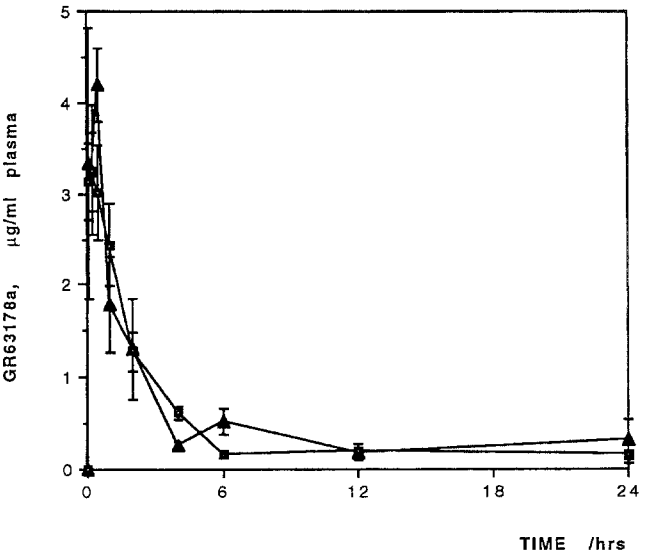


Fig. 4. Plasma drug clearance profile of GR63 178A in tumour-bearing mice after i. p. injection of 10 mg/kg drug. Profiles of BDF-1 mice with colon 38 tumour (*triangles*) and NMRI mice with MAC 26 tumour (*circles*) are shown. Values are means ± SEM of at least five mice per point

HPLC analysis was performed on a Hewlett-Packard 1090 HPLC with a diode-array detector and a HP 9000/300 chem-station for data handling and processing, as previously described [6].

In vitro cytotoxicity. The effect of GR63 178A and the metabolite GR54 374X on tumour cell growth was studied using the A2780 cell line. Cells (5×10^3/well) were seeded into 24-well culture plates in 1.0 ml RPMI + 5% fetal calf serum and allowed to attach and grow for 48 h. Depleted media was then replaced with fresh media containing a known amount of drug. After incubation at 37° C for 72 h, the media and drug was removed and the cells were washed thoroughly; this point is taken as time zero. The cells were then incubated in a further 1.0 ml fresh medium. Every second day, the cells were washed and trypsinised, and counted on a Coulter Counter (Coulter Electronics, Luton, England).

Statistics. Results were analysed for significant differences by Student's *t*-test, with logarithmic transformation of data for the growth curve values.

Results

Tumour inoculation

After an initial passage from frozen stock tumour, both mouse models had tumour inoculum take-rates in excess of 90%.

Effect of GR63 178A on tumour growth

The growth kinetics of the two murine tumour models (BDF-1 with C38 tumour and NMRI mice with MAC 26) differed slightly in terms of volume doubling times (Table 1) and also in the possession of a lag phase post-inoculation, which was not evident in the colon 38 tumour (Fig. 2) but which was apparent for up to 10 days in the

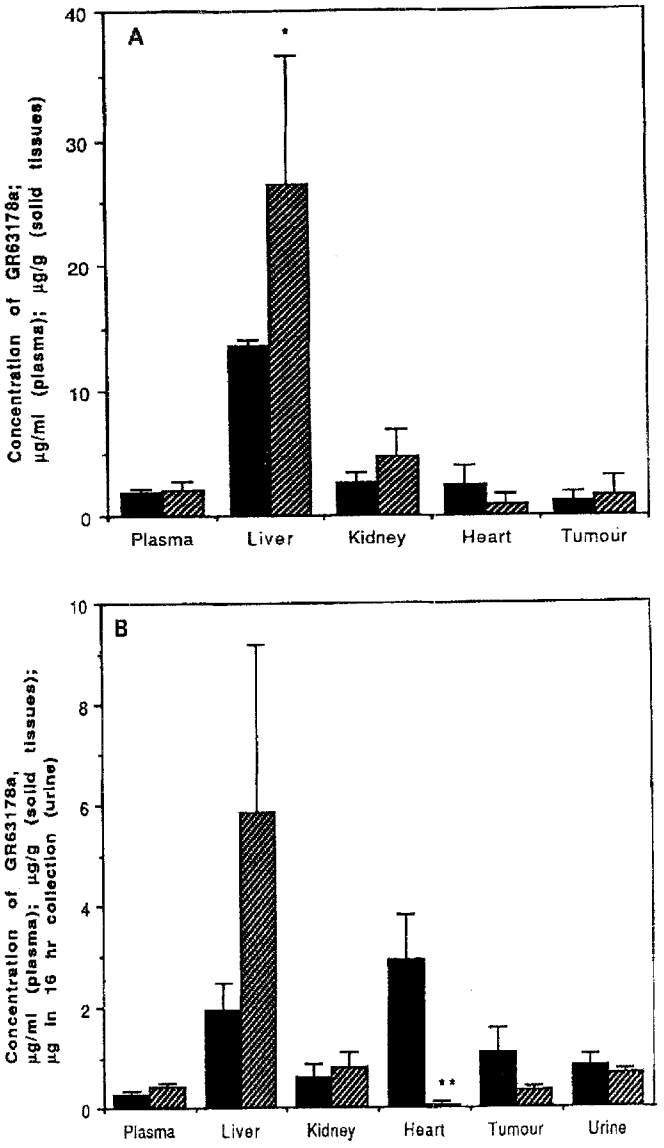


Fig. 5. Concentrations of GR63 178A in tissues of NMRI mice with MAC 26 tumour (*solid bars*) or BDF-1 mice with colon 38 tumour (*shaded bars*) at **A** 30 min and **B** 4 h after injection of the drug. Values are means ± SEM of at least four samples. Significant differences: * $P < 0.05$; ** $P < 0.01$

MAC 26 tumour (Fig. 3). The two mouse colon tumours exhibited different sensitivities to chronic administration of GR63 178A (10 mg/kg daily) over 21 days. The NMRI mouse model showed only a small reduction in the growth of the MAC 26 tumour resulting in a specific growth delay (SGD = 0.236). In contrast, the BDF-1 mouse model demonstrated significant specific growth delay in the colon 38 tumour (SGD = 1.20; Table 1). Statistically significant differences in tumour volume became apparent after more than 18 days when compared to untreated controls.

Drug disposition

Plasma drug clearance. Both mouse/tumour models showed similar plasma clearance profiles (Fig. 4). GR63 178A concentrations peaked at 15 or 30 min after injection; peak plasma concentrations were slightly higher in NMRI mice (4.195 µg/ml compared to 3.26 µg/ml in BDF-1 mice) but this was not statistically significant. Most (>90%) of the peak drug plasma concentration had been cleared by 6 h. GR63 178A was evident in plasma at a low, steady-state concentration up to 24 h post-injection. For both mice/tumour systems, the clearance profile best fitted a two compartment pharmacokinetic model. α-phase half lives were 1.1 and 0.89 h for BDF-1 and NMRI mice respectively. Total AUC values were also similar (10.059 and 12.042 h × µg/ml for BDF-1 and NMRI mice).

Table 1. Tumour doubling times and specific growth delay of MAC 26 and C38 colon adenocarcinomas in vivo when treated with GR63 178a

Mouse/tumour:	NMRI/MAC 26	BDF-1/C38
Tumour doubling time (control)	2.88	3.80
Tumour doubling time (GR63 178a, 21 days 10 mg/kg)	3.56	8.36
Specific growth delay	0.24	1.20

Plasma drug metabolism. In a separate experiment, no difference in GR63 178A levels either 30 min (peak plasma concentration time) or 4 h (closest time point to the end of α-phase and the start of β-phase elimination) post-injection were seen (Fig. 5); this was consistent with the results generated from the plasma clearance profile study (see above). However, small but statistically significant differences were seen in the levels of GR54 374X apparent in the plasmas from the two mice strains 30 min after injection, where higher concentrations were seen in the NMRI mice [0.1 ± 0.014 (SEM) µg/ml plasma] compared to BDF-1 mice [0.036 ± 0.027 (SEM) µg/ml]. At 4 h, however, no GR54 374X was detected in the plasma samples from either mouse strain (Fig. 6).

Tissues and tumour. Of the major solid tissues analysed, the greatest uptake of parent compound was in the liver of both mouse strains (Fig. 5); the liver also had the highest levels of the 9-OH metabolite (Fig. 6). Interestingly, at 30 min post-injection, the sensitive mouse model (BDF-1) had much higher levels of both the parent drug [26.404 ± 4.544 (SEM) µg/g liver] and GR54 374X [1.937 ± 0.312 (SEM) µg/g liver] when compared to the resistant NMRI livers [13.66 ± 0.42 (SEM) µg/g GR63 178A and 0.777 ± 0.059 (SEM) µg/g GR54 374X]. This difference persisted over at least 4 h after administration of the drug. The kidney similarly exhibited consistently higher levels of both GR63 178A and GR54 374X in the BDF-1 mice, but at overall lower concentrations; these differences only reached statistical significance after 4 h, however, and only in respect of GR54 374X [undetectable in NMRI mice, 0.075 ± 0.05 (SEM) µg/g kidney in BDF-1 mice]. Major differences between the two mouse strains were seen in the accumulation of both the parent drug and GR54 374X in the heart. Neither GR63 178A nor GR54 374X was seen in the hearts of sensitive mice after 4 h; in NMRI mice parent drug levels were actually higher

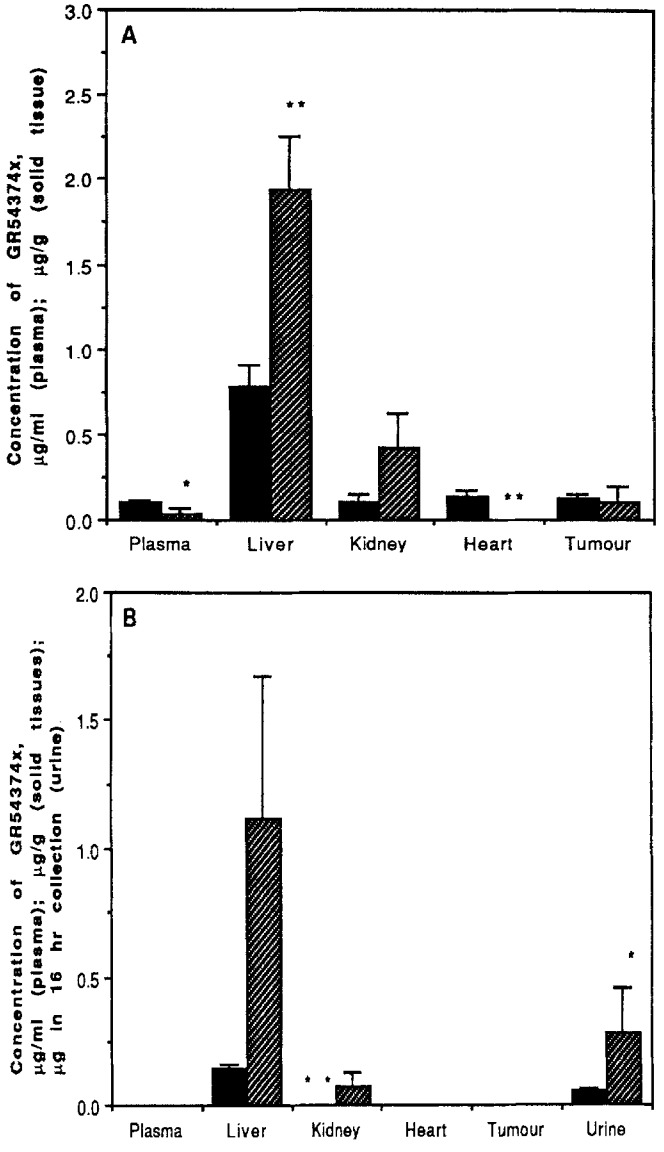


Fig. 6. Concentrations of GR54374X in tissues of NMRI mice with MAC 26 tumour (*solid bars*) or BDF-1 mice with colon 38 tumour (*shaded bars*) at **A** 30 min and **B** 4 h after injection of the drug. Values are means ± SEM of at least four samples. Significant differences, * $P<0.05$; ** $P<0.01$

at 4 h [2.93±0.9 (SEM) µg/g heart] than at 30 min [2.48±1.50 (SEM) µg/g]. GR54 374X was detectable in the hearts of NMRI mice only, and only at 30 min post-injection [0.137±0.034 (SEM) µg/g]. No obvious pathological effects due to the accumulation of either parent compound or 9-OH metabolite were apparent in these mice, however. In tumour tissue, levels of parent drug only showed apparent differences at 4 h post-injection [1.08±0.5 (SEM) in MAC 26 tumours vs 0.332±0.084 (SEM) in C38 tumours], although this did not reach statistical significance. No differences were seen in the persistently low levels of GR54 374X seen in either tumour type.

Excretion urine. There was no difference in the levels of GR63 178A detected in a 16-hour collection of urine from the two mouse/tumour models. However, the extent of excretion of the unconjugated 9-OH metabolite GR54 374X in the sensitive mouse model was significantly higher [0.278±0.181 (SEM) µg in BDF-1 mice vs 0.057±0.005 (SEM) µg in NMRI mice; Fig. 5].

In vitro cytotoxicity of GR63 178A and GR54 374X

A2780 cells grown in RPMI medium supplemented with fetal calf serum exhibited no significant increase in cell doubling time or decrease in clonogenicity as a result of pre-treatment with 10^{-7} to 10^{-4} M GR63178A or GR54374X (results not shown).

Discussion

GR63178A demonstrates antitumour activity against many tumour types in vivo, but no direct cytotoxicity is apparent in vitro. We have demonstrated inherent differences in the sensitivity of two similar murine colon adenocarcinoma models to the drug. The sensitivity of C38 tumour in BDF-1 mice supports the findings of Fenton et al. [10]. However, MAC 26 tumour in NMRI mice proved resistant to GR63178A, with minimal specific growth delay.

Potential differences in the disposition of both GR63178A and GR54374X in the two murine models, which might explain differences in drug sensitivity, were investigated. The plasma clearance profiles for GR63178A in the two model systems revealed little difference in the way the parent compound was absorbed to, or removed from, the circulation after i.p. injection. Despite the lack of a difference in circulating plasma levels, differences were seen in the tissue GR63178A concentrations in the two models, in hearts and tumours (at late time points and low levels only) and most significantly in the livers. The consistently higher concentrations of GR63178A seen in the livers of BDF-1 mice indicate a greater avidity of this BDF-1 tissue for the drug when compared to the corresponding NMRI tissue. This could lead to more extensive total hepatic metabolism in these animals, and hence the higher levels of GR54374X that were also seen in the liver, although this difference was not reflected by increased plasma levels in the BDF-1 mice, and clearly did not affect the disposition of GR54374X in the tumour, where no difference was found between the two mouse/tumour combinations. The failure of the higher hepatic levels of drug and metabolite to be reflected in the plasma may indicate a rapid clearance from the circulation (or indeed a failure for metabolite to reach the circulation if clearance was mostly biliary) and the observed differences in urinary metabolite levels would support this suggestion. From these observations it appears that either MAC 26 is inherently resistant to GR63178A or GR54374X, or that neither the parent drug nor its 9-OH metabolite are directly cytotoxic to the tumours in vivo. This latter contention is supported by our in vitro cytotoxicity data, which demonstrate a lack of cytotoxic activity through four concentration orders of magnitude. Our recent data indicates that GR63178A does not exhibit properties common to many conventional cytotoxic drugs; it is a poor redox cycler and generator of

reactive oxygen species (despite containing a quionone moiety) and does not inhibit topoisomerase II activity [7].

There would appear, therefore, to be a clear requirement for metabolic activation of this drug to produce a direct cytotoxic effect. We have previously reported the separation of six metabolites from human urine samples [6]. Mouse tissues, including tumours, analysed for this work consistently demonstrated only the parent drug GR63178A and a major metabolite GR54374X. In humans, and in murine systems where antitumour effects are seen, GR63178A is administered chronically, and this may result in the induction of systems which modulate drug handling (most obviously metabolising enzymes such as hepatic cytochrome P450s), the effect of which would not be apparent in mice receiving a single dose of the drug, as has been investigated here. The significance of this in terms of potential differences between murine and human systems in the production of ultimately cytotoxic metabolites of GR63178A has not been established.

If GR63178A and GR54374X are not directly cytotoxic to the tumour, and unidentified cytotoxic metabolites are not being produced, the parent drug's antitumour effect must be mediated by other in vivo-related mechanisms, for example immunomodulation or alteration in tumour blood flow. Response to GR63178A as an immunomodulatory stimulus might differ in the two mouse strains, leading to differences in sensitivity. The failure of GR63178A at its maximum tolerated dose to elicit an antitumour effect in five out of six ovarian tumours grown as xenografts in immune-suppressed mice [2] is evidence in favour of an immunomodulatory role in the mechanism of action of GR63178A. Similarly, the clinical observation of pain at the tumour site [15], unusual for a conventional cytotoxic, may be evidence of a tumour-related inflammatory response. Recent results provide evidence that GR63178A may indeed cause reductions in tumour blood flow [13]. Flavone acetic acid, another polycyclic quinone-containing antitumour agent, is also known to cause changes in tumour blood flow [12, 17] and is similar to GR63178A in many respects; it is inactive in in vitro cytotoxicity assays against murine P388 and L1210 leukaemias [4], and in animals it has a similarly high therapeutic index to GR63178A [1].

The work we have presented here demonstrates that, after a single dose of GR63178A, differences in the disposition of GR63178A and GR54374X in normal tissues arise in mice implanted with colon tumours of varying sensitivity to the parent drug. We are unable to correlate differences in sensitivity to significant differences in tumour concentrations of parent drug or metabolite (either when plasma concentrations of GR63178A reach a peak, or when α-phase drug elimination has ended) despite large differences in hepatic disposition. This suggests that the mechanisms of action of GR63178A may be mediated by further metabolism. Metabolism to GR54374X would seem to be an unlikely mechanism of cytotoxicity, as this 9-OH metabolite appears to be inactive in vitro. However, the metabolism of the parent compound to an as yet unidentified cytotoxic product (possibly via a 9-OH intermediate) cannot be eliminated. Alternatively, differences in sensitivity may be related to factors other than direct drug handling, such as changes in tumour blood flow, or immunomodulatory effects. These factors remain to be investigated.

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