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Adequate Tumour Quinidine Levels for Multidrug Resistance Modulation can be Achieved *in vivo*

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The multidrug resistance (MDR) phenotype can be reversed *in vitro* by a number of agents thought to interact with P-glycoprotein (P-gp). Although plasma levels, adequate for MDR modulation, can be achieved with certain modulators, concern has been expressed that tumour levels may be inadequate due to high plasma protein binding. Mice bearing an MDR-positive human tumour xenograft were injected intraperitoneally with quinidine (150 mg/kg). After 2 h the mean plasma quinidine level was 1.9 μg/ml (5.1 μmol/l) and the mean tumour quinidine level was 6 μg/g. Thus tumour levels were higher than plasma levels and were within the range known to be effective *in vitro*. Three tumour biopsy specimens were obtained from patients who had received oral quinidine prior to surgery. Plasma and tumour levels were similar and were comparable with those measured in mice. This study should dispel fears of inadequate tumour levels of this and other modulators due to high plasma protein binding and encourage future clinical trials of modulators in MDR-positive human tumours.
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INTRODUCTION

THE DEVELOPMENT OF resistance to cytotoxic drugs is a major problem in the treatment of cancer. One mechanism of resistance, multidrug resistance (MDR), has been extensively studied in both animal [1] and human [2] cell lines *in vitro* and is characterised by crossresistance to a number of drugs following exposure to only one of them. The drugs involved in the MDR phenotype are anthracyclines, vinca alkaloids and etoposide [3]. Although these drugs are not structurally related, and share different intracellular targets, they are all hydrophobic compounds derived from natural products and they appear to share a common mechanism of resistance. This cross-resistance is related to the presence of a specific 170 000 dalton transmembrane protein named P-glycoprotein (P-gp) [4]. P-gp is encoded by a specific human gene, *mdr*-1 [5] and is thought to act as an energy-dependent drug efflux pump [6].

A number of compounds have been shown to modulate MDR *in vitro*, including quinidine [7], verapamil [8], amiodarone [9] and, more recently, cyclosporin [10]. There is some evidence to suggest that some of these drugs may bind to P-gp [11] and bring about reversal of MDR by acting as a competitive substrate for the drug efflux pump [12].

Although there are many compounds which do circumvent MDR *in vitro*, their clinical potential is often limited by the inability to achieve plasma levels, adequate for MDR modulation *in vitro*, which do not cause adverse toxicity. As a result of dose-limiting hypotension and heart block in patients [13] the plasma levels of verapamil achieved without toxicity are several fold lower than the most effective concentration *in vitro* [14]. In contrast, adequate plasma concentrations of quinidine, an effective modulator of MDR *in vitro*, can be achieved in breast cancer patients without toxicity following a 4-day course of oral quinidine [15].

One further problem in attempting to modulate MDR *in vivo* is that many of the *in vitro* modulators of MDR are highly protein bound in plasma, e.g. quinidine [16], and it has been suggested that this may lead to quinidine tumour levels inadequate for circumvention of MDR. Furthermore, with increased levels of the acute phase plasma protein alpha-1 acid glycoprotein in many cancer patients [17], which has been shown to bind a number of drugs including quinidine [18], the free plasma quinidine may be decreased even further.

We have therefore measured simultaneous plasma and tumour quinidine levels, by spectrofluorophotometry in an animal model of MDR, to try to confirm quinidine concentrations adequate for MDR modulation in tumour as well as plasma. In order to identify a range of quinidine concentrations which are effective in modulating MDR *in vitro* a MDR-positive cell line ($MCF7^{ADR}$) was exposed to quinidine, in combination with epirubicin, in a microtitration cytotoxicity assay (MTT). In addition we have also measured tumour quinidine levels in three human breast cancers in patients pretreated with oral quinidine sulphate for 4 days prior to surgery.

MATERIALS AND METHODS

Animals and cell lines

The human ovarian carcinoma cell line 2780AD, known to express P-gp [19], was obtained from R.F. Ozols (National Cancer Institute, Bethesda, USA). This cell line was obtained from the parental cell line A2780 by chronic exposure to doxorubicin *in vitro*. The cell line was maintained in RPMI 1640 culture medium supplemented with glutamine 2 mmol/l (Gibco), 10% fetal calf serum (Globepharm, Surrey, England) and insulin (0.25 U/ml). The resistance of this line was maintained by growth in the presence of doxorubicin (2 μmol/l).

The 2780AD cell line was grown as a xenograft in female MF1 *nu/nu* athymic mice by subcutaneous inoculation of approximately 10^6 cells in an injection volume of 0.5 ml phosphate-buffered saline (PBS). Following establishment of the

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xenograft 3 weeks later the tumour was passaged into a further 10 mice and allowed to grow. The mice were kept at a constant temperature using a 12 h light/dark cycle with free access to food and water.

The human breast carcinoma cell line $MCF7^{ADR}$ was used as an *in vitro* model of MDR. This cell line was derived from the drug-sensitive parent line MCF7 by chronic exposure to doxorubicin and both cell lines were obtained from K. Cowan (National Cancer Institute, Bethesda, USA). The cell line was maintained in RPMI 1640 medium with supplements, as for the 2780AD cell line.

Drug administration and sampling

All mice were weighed and given a bolus intraperitoneal injection of quinidine sulphate (Sigma) at the maximum tolerated dose (150 mg/kg) made up at a concentration of 3.75 mg/ml in PBS. Two hours postinjection, the mice were killed, blood was obtained by cardiac puncture and plasma stored in 200 μl aliquots at −20°C. In addition, the subcutaneous tumours were excised, weighed and then stored at −70°C until assayed.

MTT assay

This assay has been described in detail previously [20]. In brief, $MCF7^{ADR}$ cells were plated out in 96-well microtitration plates (Linbro, Rickmansworth, UK), at a density of 10^3 per well, and allowed to attach and grow for 3 days. The medium was then removed from the wells and replaced with 200 μl of fresh medium containing epirubicin with or without quinidine. A serial dilution of eight concentrations of epirubicin was prepared at twice the final concentration. These were then diluted with quinidine at twice the final concentration of quinidine prior to addition to the wells. Four wells were used for each epirubicin concentration, in combination with each of three quinidine concentrations (3.3 μmol/l, 6.6 μmol/l, 9.9 μmol/l), and the cells were also exposed to epirubicin alone. After a 24-h exposure to drug the cells were fed daily with fresh medium for 3 days.

On the fourth day the cells were fed with medium and MTT (50 μl, 5 mg/ml) was added to each well. Plates were incubated in the dark at 37°C for 4 h, the MTT and medium removed and the MTT-formazan product dissolved in dimethyl sulphoxide (200 μl/well). Glycine buffer (25 μl/well, 0.1 mol/l, pH 10.5) was added and the absorbance measured at 570 nm in a multiwell plate reader (Bio-rad). The results were expressed as the epirubicin concentration required to kill 50% of the cells (ID_{50}).

Patients' details

3 patients undergoing breast surgery at the University Department of Surgery, Royal Infirmary, Glasgow, were treated with oral quinidine bisulphate (Astra, Kings Langley, England) 250 mg twice daily for 4 days prior to surgery (total dose 2 g) to achieve a steady-state level of quinidine. All 3 patients gave informed consent to this study. Following excision of the tumour, 3–4 h after the last dose of quinidine, a representative sample was snap frozen in liquid nitrogen and stored at −70°C until assayed.

Quinidine assay

The quinidine content of plasma and tumours (murine and human) was measured using a spectrofluorophotometric technique which relies on the ability of quinidine to fluoresce in an acid medium. Extraction was performed according to the method of Cramer and Isaakson [21] with the modification that quinidine was extracted into n-heptane instead of benzene [16]. The technique has been validated both in terms of reproducibility and recovery and the error associated with measurements is < 5%.

Plasma quinidine was extracted from a working volume of 1 ml human plasma or 200 μl murine plasma made up to 1 ml with distilled water. NaOH (0.5 ml 0.2 mol/l) and NaCl (0.5 g) were added to the sample and the quinidine then extracted into 2.5 ml n-heptane containing 3% isoamylalcohol. Sulphuric acid (1 ml, 0.1 mol/l) was added to 2 ml of the organic phase and the fluorescence of the aqueous phase was measured in a Shimadzu RF-540 recording spectrofluorophotometer. In addition, a 1 ml sample of human plasma spiked with 10 μg quinidine sulphate was used as a recovery control and a normal plasma was used as a negative control.

All tumour samples were homogenised in 5 ml sulphuric acid (0.1 mol/l) and each batch processed included a recovery and a negative control. The homogenates were added to 0.2 ml 1.5 mol/l NaOH and 0.5 g NaCl and the extraction then proceeded as for plasma. The results were corrected for the extraction of the spiked sample and plasma and tumour levels are expressed in μg/ml and μg/g, respectively. In order to compare plasma and tumour levels, we have equated 1 g of tumour to 1 ml of water and calculated a tumour/plasma extraction ratio.

Table 1. Sensitivity of cells

Drug(s)	ID_{50} (μmol/l)	Modulation*
Epirubicin alone	33.60 (4.16)	—
Epirubicin plus quinidine		
3.3 μmol/l	2.15 (1.23)	16-fold
6.6 μmol/l	1.50 (0.17)	22-fold
9.9 μmol/l	0.67 (0.21)	50-fold

Mean (S.E.).
* The increased sensitivity with each concentration of quinidine.

RESULTS

MTT assay

The sensitivity of $MCF7^{ADR}$ cells to epirubicin alone and in combination with quinidine at three different concentrations (3.3 μmol/l, 6.6 μmol/l and 9.9 μmol/l) is shown in Table 1. This shows quinidine to be an effective modulator at all three concentrations used in this experiment.

Quinidine concentrations in mice

Plasma and tumour quinidine concentrations in 10 tumour-bearing nude mice are shown in Table 2. One plasma sample was lost during the extraction procedure due to a breakage. The mean plasma quinidine concentration was 1.94 μg/ml (range 0.21–4.01 μg/ml). This represents a mean plasma concentration of 5.13 μmol/l (0.55–10.58 μmol/l). The mean tumour quinidine concentration was 6.04 μg/g (2.46–12.24 μg/g). The tumour/plasma extraction ratios were all greater than 1.0 (1.20–33.5) and the recovery of the spiked samples in plasma and tumour was 55% and 77%, respectively.

Human quinidine concentrations

The results of both plasma and tumour quinidine concentrations in 3 patients with breast cancer are shown in Table 3. The mean plasma concentration was 1.48 μg/ml (1.05–1.84 μg/ml) which represents a mean concentration of 4.54 μmol/l

Table 2. Plasma and tumour quinidine concentrations (μg/g) in 10 tumour-bearing mice after quinidine sulphate injection

Animal	Plasma (μmol/l)	Plasma (μg/ml)	Tumour (μg/g)	Tumour/plasma extraction ratio
1	8.04	3.05	4.95	1.63
2	—	—	12.24	—
3	4.20	1.59	2.46	1.55
4	4.30	1.63	8.95	5.49
5	10.58	4.01	5.87	1.46
6	9.11	3.45	5.53	1.60
7	7.93	3.01	3.61	1.20
8	0.55	0.21	7.04	33.50
9	0.62	0.24	6.21	26.40
10	0.82	0.31	3.55	11.45

(3.22–5.65 μmol/l). The mean tumour concentration was 1.39 μg/g (1.25–1.46 μg/g). The mean tumour/plasma extraction ratio was 1.0 (0.68–1.39) and the recovery of the spiked samples was 99% (plasma) and 86% (tumour).

DISCUSSION

These results show that tumour concentrations of the multidrug resistance modulator, quinidine, adequate for circumvention *in vitro*, can be achieved in both an animal model of MDR and in patients with breast cancer. In all 10 tumour-bearing mice, the tumour concentration was greater than the plasma quinidine and although these figures represent the level at one time point only (2 h postinjection) they nevertheless demonstrate that adequate tumour levels for MDR modulation are achievable. This concurs with a previous pharmacokinetic study of quinidine in the mouse which found high tissue accumulation in lung, liver and heart despite a 77% binding to mouse blood constituents [16].

The results of both plasma and tumour concentrations in patients with breast cancer show levels that are lower than the most effective concentration (6.6 μmol/l) in MCF7 cells exposed to doxorubicin *in vitro* [22]. Our results however show quinidine to be effective *in vitro*, in combination with epirubicin, even at a concentration of 3.3 μmol/l and this was attained in all three human tumour specimens according to the tumour/plasma extraction ratios. Although a previous study in patients with breast cancer has shown that oral quinidine bisulphate 250 mg twice daily for 4 days can achieve mean plasma steady state levels of 5.6 μmol/l [15] there was a wide range of concentrations (2.1–22.1 μmol/l) in that study. Our results, although slightly less than the mean steady-state plasma level achieved in that study, are within this range of concentrations. Despite the fact that Jones *et al.* used a radioimmunoassay to measure quinidine, in contrast to the spectrofluorophotometric technique used by ourselves, a comparison of the two techniques has failed to reveal any major differences when samples are assayed by both techniques (results not shown). These results once again demonstrate that plasma and tumour quinidine concentrations are of a similar magnitude.

Table 3. Plasma and tumour quinidine concentrations in 3 patients with breast cancer pretreated with oral quinidine sulphate prior to surgery

Patient	Plasma (μmol/l)	Plasma (μg/ml)	Tumour (μg/g)	Tumour/plasma extraction ratio
1	3.22	1.05	1.46	1.39
2	4.76	1.55	1.45	0.93
3	5.65	1.84	1.25	0.68

In conclusion, our results provide the first documented evidence of tumour quinidine concentrations, in both murine xenografts and human breast cancers, in the range of concentrations known to circumvent multidrug resistance *in vitro*. It is hoped that this will allay fears of inadequate tumour concentrations of this and other modulators due to high plasma protein binding. Furthermore, it is known that the dose of quinidine used in patients in this study (250 mg twice daily for 4 days) can be tolerated without toxicity in breast cancer patients. Quinidine is therefore currently being assessed as a potential modulator in a phase III, placebo-controlled, double-blind trial in combination with epirubicin in patients with advanced breast cancer and the results are awaited.

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In vitro Growth Ability and Chemosensitivity of Gastric and Colorectal Cancer Cells Assessed with the Human Tumour Clonogenic Assay and the Thymidine Incorporation Assay

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A human tumour cloning assay (HTCA) has been performed on 191 samples of gastric and 152 samples of colorectal cancers, and a thymidine incorporation assay (TIA) on 178 samples of gastric and 109 samples of colorectal cancers. The rate of evaluable assays was significantly higher in the TIA than in the HTCA ($P < 0.01$). In terms of *in vitro* growth potential in the two assays, gastric cancer cells were less active than the colorectal cancer cells ($P < 0.05$). In frequency of *in vitro* sensitivity to drugs, gastric cancer was more chemosensitive than colorectal cancer in both assays. The *in vitro/in vivo* correlations of high resistance-predictive ratios and low sensitivity-predictive ratios were similar in both assays. The results indicate that the TIA is more applicable than the HTCA to screening of active agents against fresh gastrointestinal cancers.
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CHEMOSENSITIVITY ASSAYS using tetrazolium dyes (MTT assay) have been used in the field of haematological malignancies [1–3] and a modified form of this is currently being successfully applied by the National Cancer Institute USA to the chemosensitivity testing of new drugs on cell lines [4]. However, when it is applied to solid tumours like gastrointestinal cancers, a formazan product from viable fibroblasts and other non-malignant cells cannot be excluded at the assessment [5].

The human tumour clonogenic assay (HTCA) using a soft agar culture system independently developed by Hamburger and Salmon and by Courtenay *et al.* has been shown to be suitable for culturing a variety of solid tumours [6, 7]. In this study, the HTCA was based on the method of Hamburger and Salmon. In spite of the early favourable reports of the HTCA, its practical value for predictive testing has been the focus for debate, chiefly because of the insufficient rates of evaluable assays [8–16].

To attain high evaluability rates we developed the thymidine incorporation assay (TIA) in which a two-layer soft agar culture system was used as well as in the HTCA [17–21]. In addition, a wide range of assay evaluability rates for different tumour types have been observed [10], which means that individual assay evaluability of tumour specimen should be studied on the selected tumour. In this study, therefore, the organs with tumour studied have been restricted to the stomach and the large bowel, and their assay evaluabilities and chemosensitivities were comparatively assessed with the HTCA and the TIA.

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MATERIALS AND METHODS

Tumour collection

Solid tumour specimens were obtained by surgery and immediately placed in Chee's modification of essential medium (CEM)(MA Bioproducts, Walkersville, USA) supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin (both from Gibco) and 15% heat-inactivated fetal calf serum (Flow).