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RAPID COMMUNICATIONS

The sensitivity of human tumour cells to quinone bioreductive drugs: What role for DT-diaphorase?

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Abstract

15 human tumour cell lines (lung, breast and colon) have been evaluated for their sensitivity to the quinone based anti-cancer drugs Mitomycin C, Porfiromycin, and EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(IH-indole-4,7-dione)prop-β-en-α-ol). Sensitivity has been compared with the intra-cellular levels of DT-diaphorase, an enzyme thought to be important in the reductive activation of these quinones. No correlation exists between levels of DT-diaphorase and sensitivity to Mitomycin C or Porfiromycin. However, for EO9 those cell lines showing highest levels of DT-diaphorase activity tend to be the most sensitive.

Introduction

Bioreductive drugs are compounds that can selectively target hypoxic tumour cell populations resistant to both radiation and to drug-based therapies. The leading compounds are RB 6145 and SR 4233, which are scheduled for Phase I clinical trial in 1992 [1]. Some of the pathways by which reductive activation of these drugs can occur have been identified. These include: cytochrome P450 (various isozymes), cytochrome P450 reductase, xanthine oxidase, etc. Under normal aerobic conditions, initial reduction via these systems will occur but these are one-electron reductions which are potentially reversible by oxygen, resulting in comparatively inconsequential futile redox cycling. In contrast, under anaerobic conditions reduction can proceed further to yield highly cytotoxic species. Mitomycin C (MMC) and related quininoid compounds, such as the analogue Porfiromycin (POR) and the indoloquinone EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(IH-indole-4,7-dione)prop-β-en-α-ol) [2] (which is also scheduled for clinical trial in 1992), similarly require reductive activation. One pathway by which this can occur is via a concerted two-electron process, which is oxygen-independent and is carried out by DT-diaphorase (NAD(P)H:(quinone acceptor)oxidoreductase(EC1.6.99.2)). This is one reason why it is generally thought that MMC has less selectivity for killing hypoxic relative to oxic cells, when compared to other agents. The role of DT-diaphorase in cancer chemotherapy has recently been reviewed by Riley and Workman [3]. The importance of this enzyme in determining MMC toxicity has often been inferred from studies in human and rodent cells with sub-lines which have altered levels of DT-diaphorase. However, there has not yet been any systematic study of the requirement for DT-diaphorase for expression of sensitivity to quinone anti-cancer drugs in human tumour cells. This paper describes the sensitivity of 15 human tumour cell lines to MMC, POR and EO9 and compares this to intra-cellular levels of DT-diaphorase.

Materials and Methods

Table 1 lists the human tumour cells used in this work. The lung lines are representative of the major histological types seen clinically, and the breast lines express a range of hormone and growth factor receptor phenotypes, e.g. MDA 468 is oestrogen receptor (ER) negative whereas T47D and ZR75 are ER positive. All cell lines were maintained in exponential growth phase in RPMI 1640 medium supplemented with 10% foetal bovine serum and 2mM glutamine. Exponentially growing cells were harvested and seeded into 96-well microtitre plates at an appropriate density that would allow exponential growth for 4 days. Drugs were added, in replicates of 4 and 8 for adherent and floating cells respectively, at appropriate concentrations and cells incubated at 37°C (5% CO_2, 100% humidity) for 4 days. Cytotoxicity was then determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5;diphenyltetrazolium bromide) assay [4]. MTT (50 μl at 2 mg/ml) was aliquoted into the wells and the cells

Table 1
Human tumour cells, their levels of DT-diaphorase and values of IC_{50} when exposed to quinone-based bioreductive drugs

Cell line	Origin	Histological type	DT-diaphorase activity	IC_{50}/nM		
				MMC	EO9	POR
MDA468	Breast	Adeno	13	87	2060	1430
T47D		Adeno	46	880	5650	3920
ZR75		Adeno	2290	30	680	1270
A549	Lung	Adeno	5930	160	7.5	110
H226		Squamous	1040	920	120	2330
H249		SCLC	5	1120	4080	650
H322		Bronchio-alveolar	5030	280	20	1130
H358		Bronchio-alveolar	-	460	22	1960
H417		SCLC	120	120	270	220
H460		Large cell	5340	8	7	3
H522		Adeno	240	134	33	290
H526		SCLC - variant	19	62	210	260
H647		Adeno squamous	5140	25	12	75
H841		SCLC - variant	450	290	82	1410
HT29	Colon	Adeno	2760	380	160	700

incubated for a further 4 hours. Plates of adherent cells were inverted to remove medium and unconverted MTT, while medium from floating cells was aspirated leaving ~30µl per well. Formazan crystals were solubilized with DMSO plus glycine buffer (0.1M glycine in 0.1M NaCl, pH 10.5 [5]) and optical densities determined at 540nm using a Titertek Multiskan Plus MKII ELISA plate reader. Data were analysed using Deltasoft Elisa Analysis Software (BioMetallics Inc., Princeton, N.J.). Values of IC_{50}, the concentration of drugs required to reduce optical density to 50% of control, were used as the measure of cellular sensitivity to a given drug. MMC, EO9 and POR were gifts from Kyowa, via Farillon Pharmaceutical Distribution, Romford, Essex; the EORTC NDDO and the DCT-NCI, U.S.A. respectively. All drugs were dissolved in DMSO. For measurement of enzyme activity cells were harvested, pooled and centrifuged. Pellets were washed twice in ice cold PBS and finally resuspended in a hypotonic buffer (10mM HEPES/KOH pH 7.9, 1.5mM MgCl, 10mM KCl, 0.5mM DTT). The suspensions were homogenised and centrifuged at 4000 rpm for 10 minutes (4°C). Supernatants were flash frozen and stored in liquid nitrogen. DT-diaphorase activity was measured at 37°C using menadione as substrate and cytochrome C as terminal electron acceptor [6]. Activity was calculated as that fraction which was inhibited by dicoumarol. Values quoted are nmoles Cytochrome C reduced per minute per mg protein.

Results

Values of IC_{50} were calculated from each of, at least, three replicate experiments and the mean values derived with each drug for each of the cell lines are given in Table 1. Values of IC_{50} for cell lines exposed to MMC are plotted against IC_{50} for POR in Figure 1. The line is the best fit obtained by regression analysis of the data, which gives a correlation coefficient of 0.83 and slope of 1.4 ± 0.24 (significance of slope: $t_{13} = 5.84$ [$P = 0.00006$]). While it would be expected that the mode of activation of POR would be similar to MMC (7), the regression analysis implies that POR is less effective than MMC in the more resistant cell lines. It is likely that the spectrum of adducts formed and their repair will be critical in determining relative cellular sensitivity to MMC and POR (8-10). The activity of MMC correlates less well with EO9 ($r = 0.56$; slope $= 2.38 \pm 0.83$, $t_{13} = 2.87$ [$P = 0.013$]). This suggests that differences exist between the two compounds with regard to their mechanism of bioactivation and/or subsequent mode of action.

Measurements of the levels of the enzyme DT-diaphorase have been made in the human tumour cells and the results are shown in Table 1. No correlation exists between DT-diaphorase activity and values of IC_{50} for MMC or POR. However, there is a trend for high DT-diaphorase activity to be accompanied by sensitivity to EO9 and this is illustrated in Figure 2.

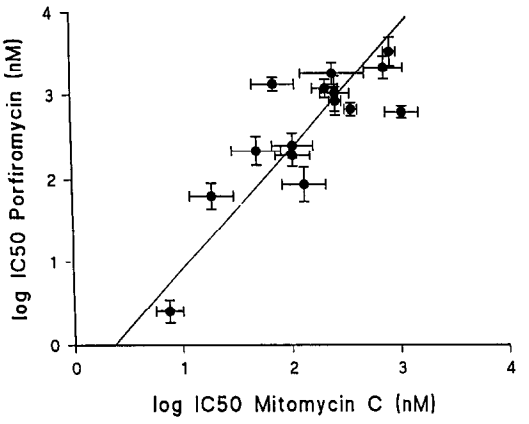

Figure 1: Values of IC_{50} derived from the MTT assay for human tumour cells exposed to Mitomycin C and Porfiromycin. Each data point represents mean values ± s.e. for a single cell line.

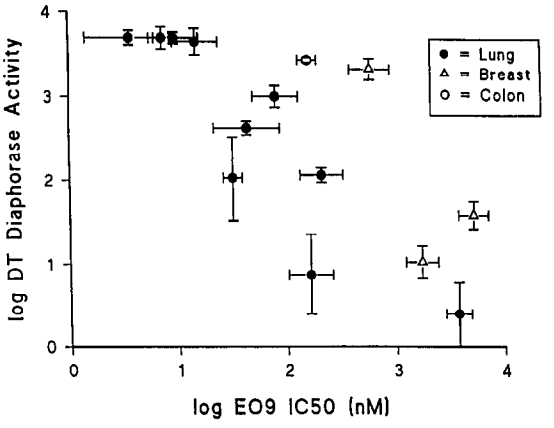

Figure 2: Values of IC_{50} derived from the MTT assay for human tumour cells exposed to EO9 plotted as a function of DT-diaphorase activity in each cell line. Values ± s.e. are derived from at least three separate determinations.

Discussion

Part of a strategy to rationally develop bioreductive drugs is to gain an understanding of the molecular enzymology of bioreductive activation. This will allow exploitation of reductase specificity in order to target appropriate agents to particular human tumours based on their enzymology. This enzyme-directed approach [11] may be applicable for MMC, with DT-diaphorase being the key enzyme for reductive activation in air. The importance of DT-diaphorase in the expression of the cellular toxicity of MMC was initially suggested by Keyes *et al.* [12]. Work with pairs of cell lines with differing levels of DT-diaphorase showed MMC sensitivity to be associated with high DT-diaphorase activity [13,14]. Further, loss of DT-diaphorase from CHO-AA8 cells [15] and non-transformed human skin fibroblasts from a cancer prone family renders the cells resistant to MMC and POR [7,16]. In contrast, Pristos *et al.* [17] measured DT-diaphorase levels in three solid tumour xenografts, in nude mice, prior to treatment with MMC and found an inverse relationship between drug sensitivity and enzyme activity. It is now apparent that MMC is only a poor substrate for DT-diaphorase at physiological pH [18], although metabolism of MMC by DT-diaphorase can be enhanced at acid pH [13]. However, what the significance of this is in terms of the intracellular mileau is unclear. The notion that high enzyme activity is likely to be predictive for MMC toxicity in air is equivocal, which is clearly apparent from the results reported in this paper. By the very nature of the assay we have used to assess toxicity (continuous exposure) it is conceivable that DT-diaphorase expression may change during treatment (see, e.g. 17). It is known that the expression of DT-diaphorase can be altered by a variety of treatments [19] and this may contribute to the poor relationship shown here between initial cellular levels of DT-diaphorase activity and subsequent sensitivity to MMC and POR. EO9 is metabolized much more effectively than MMC by DT-diaphorase [20]. This finding led to the suggestion [3] that tumour cells with high levels of DT-diaphorase will be a good target for treatment with EO9. Clearly, we have shown there to be a trend for those cell lines showing highest levels of DT-diaphorase activity to be the most sensitive to EO9. Within the panel of cell lines there may be a difference between tumour types, with breast cancer cells being more resistant to EO9 than the lung lines for a given DT-diaphorase activity, but more work is needed to confirm this. MMC shows only modest differential toxicity towards hypoxic cells when compared to bioreductive drugs such as SR 4233 and RSU 1069/RB 6145 [21]. This has been associated with the latter drugs being primarily activated by one electron reducing enzymes [22]. However, EO9 also shows substantial activity towards hypoxic relative to aerobic murine KHT sarcoma cells *in vitro* and *in vivo* [23]. This indicates that a compound such as EO9 may not only be useful for targetting aerobic tumour cells rich in DT-diaphorase but also in dealing with radio- and chemo-resistant hypoxic cells in solid tumours.

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