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Differential properties of cisplatin and tetraplatin with respect to cytotoxicity and perturbation of cellular glutathione levels

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Summary. We compared the cytotoxicity and the effects on the levels of cellular glutathione (GSH) of cisplatin and tetraplatin in the mouse mammary tumour cell line EMT6/P and a cisplatin-resistant variant, EMT6/CPR. EMT6/CPR showed a 2.5-fold resistance to cisplatin but no cross-resistance to tetraplatin. Basal cellular GSH levels in the resistant line were 1.7-fold those in the parent cell line. On exposure of cells to cisplatin, cellular GSH levels initially dropped in both cell lines but subsequently rose. At between 1 and 4 h after the onset of drug exposure, GSH levels were up to 2-fold the baseline levels. The cisplatin-resistant subline displayed higher GSH levels than the parent cell line at all time points. Tetraplatin failed to influence cellular GSH levels in either of the cell lines.

Introduction

Cisplatin is a highly effective agent in the curative treatment of a number of tumours, including testicular cancer and ovarian carcinoma [15]. However, its therapeutic efficacy is limited due to the emergence of drug-resistant cells [2]. Several mechanisms of cellular resistance to cisplatin have been described, including a reduction in cisplatin influx [13], enhanced DNA repair [12] and elevated glutathione (GSH) levels [7].

The role of GSH and related enzyme activities in the detoxification of chemotherapeutic agents is widely acknowledged [4]. Elevation of GSH levels using 2-oxothiazolidine-4-carboxylate has been shown to increase the resistance of normal human lung fibroblasts to cisplatin [19]. Conversely, depletion of GSH enhances the cytotoxicity of cisplatin in some cell lines [9]. A 2- to 3-fold elevation in GSH levels is often, albeit not always, observed in cisplatin-resistant cell lines [2]. Recently, Meijer et al. [14] observed changes in cellular GSH content in sensitive and resistant human small-cell lung-cancer cell lines after exposure to cisplatin. Whereas the GSH content in the sensitive cell line increased continuously over a 4-h period, it changed only slightly in the resistant line.

The ability of platinum-containing analogues to circumvent cisplatin resistance has also been studied [10]. Many platinum complexes are now available for preclinical analysis and some have entered clinical trials. Carboplatin and *cis*-dichloro-*trans*-dihydroxy-bis-isopropylamine platinum(IV) (CHIP) have been shown to be less nephrotoxic than cisplatin [6]. However, cisplatin-resistant cell lines often show cross-resistance to both of these compounds [10]. Platinum complexes based on 1,2-diaminocyclohexane(1,2-DACH) such as *trans*-tetrachloro-1,2-diaminocyclohexane platinum(IV) (tetraplatin) promise not only to reduce nephrotoxicity [20] but also to circumvent cisplatin resistance. Anderson et al. [1] reported that tetraplatin retained activity in cisplatin-resistant P388 leukaemia cell lines. Also, Hills et al. [10] found differential sensitivity for human ovarian carcinoma cell lines to various cisplatin analogues. Whereas the profiles of the sensitivity of these cell lines to cisplatin and carboplatin were similar, the profile of tetraplatin sensitivity was completely different. It is not known whether these response patterns relate directly to mechanisms of resistance such as elevated cellular GSH levels. We therefore studied the cytotoxicity and the effect on levels of GSH of both cisplatin and tetraplatin in the sensitive mouse mammary tumour cell line EMT6/P and its cisplatin-resistant counterpart EMT6/CPR.

Materials and methods

Chemicals. Cisplatin and tetraplatin were kindly supplied by Dr. L. Kelland, Institute of Cancer Research, Sutton, Surrey.

Cell lines and preparation techniques. The murine mammary tumour cell line designated EMT6/Ca/VJAC, hereafter referred to as EMT6/P, origi-

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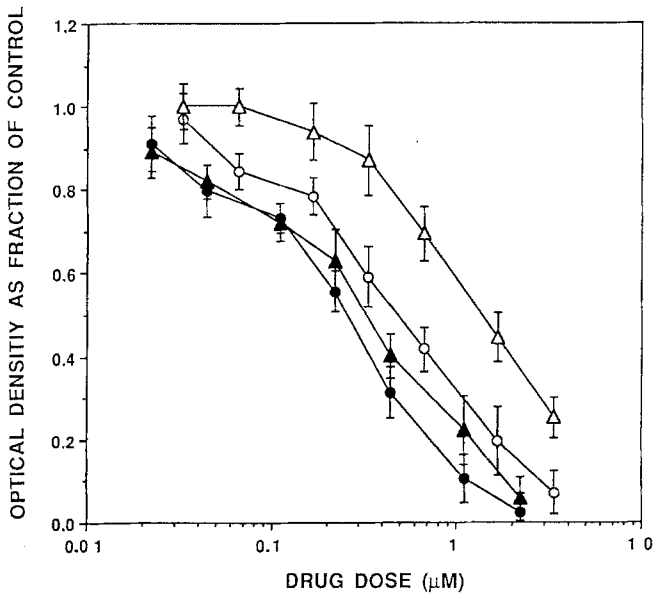

Fig. 1. Chemosensitivity of EMT6/P (*circles*) and cisplatin-resistant EMT6/CPR (*triangles*) to cisplatin (*open symbols*) and tetraplatin (*closed symbols*) as assessed by MTT assay

nated in a mouse alveolar tumour nodule and was successively transplanted into animals and in vitro culture [17] before being grown in continuous culture. Cells were grown as monolayers on plastic in Eagle's minimal essential tissue-culture medium supplemented with 20% newborn calf serum plus antibiotics. Cultures were maintained at 37° C in an atmosphere containing 8% CO_2 and 92% air. The drug-resistant variant of this cell line, EMT6/CPR, was derived by continuous in vitro exposure to increasing concentrations of cisplatin over a period of 6 weeks. The final concentration achieved was 0.83 μM cisplatin, and the EMT6/CPR subline is routinely maintained in this concentration. Subculture was carried out by 15 min exposure to 0.1% trypsin solution in phosphate-buffered saline (PBS). For experiments, 2×10^5 cells were set up in 25-cm^2 flasks. EMT6/CPR cells were set up without drug in the medium. After 3 days, cells were harvested in the logarithmic phase of growth and were used either in the tetrazolium dye (MTT) assay (see below) or for determination of drug effects on GSH levels. Population-doubling times of EMT6/P and EMT6/CPR (in the absence of cisplatin) during exponential growth were 12 and 15 h, respectively. The DNA content/cell was similar in the two lines. After preparation of a single-cell suspension, 5×10^5 cells were set up in 10-ml tubes in a volume of 500 μl medium. A further 500 μl medium containing twice the required final concentration of cisplatin or tetraplatin was then added and the tubes were incubated at 37° C in an atmosphere comprising 92% air and 8% CO_2. After various incubation periods, the cells were centrifuged, resuspended in PBS and lysed for GSH determination.

MTT assay. The MTT assay was carried out as previously described by Mosmann [15], with a number of modifications [22]. This assay is based on the ability of living cells to reduce a yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to an insoluble purple formazan precipitate. The assay is carried out in 96-well microtitre plates. Cells were inoculated onto the plates using 200 μl/well of a cell suspension containing 3×10^3 cells/ml, and drugs dissolved in PBS were added in a volume of 20 μl. After incubation of cells with cisplatin or tetraplatin, the colorimetric endpoint is determined spectrophotometrically. A linear relationship between the number of cells plated and the resulting absorbance can be observed after 3 days.

On day 3, 20 μl of a 5-mg/ml solution of MTT in PBS was added to each well and the plates were returned to the incubator for a period of 5 h. At the end of this time, the medium was removed from each well, 200 μl dimethylsulphoxide was added to dissolve the crystals and the plates were shaken for 10 min on a plate shaker. The absorbance in the wells was read on a dual-wavelength Titertek Multiskan MCC 340 plate reader using wavelengths of 540 and 690 nm. The output from the plate reader was processed automatically by a BBC model B microcomputer. In all such experiments, four replicate wells were used for each concentration. The dose of drug required to reduce the absorbance to 50% of the control level was defined as the ID_{50}.

GSH assay. The spectrophotometric determination of reduced and oxidized GSH was carried out as described by Tietze [21]. This assay is based on the reaction of free GSH with Ellman's reagent [5,5′-dithio-bis-(2-nitro-benzoic) acid, DTNB], producing a change in absorbance at 412 nm. The assay involves enzyme recycling of oxidized GSH mediated by glutathione reductase. Cells resuspended in 900 μl PBS were lysed and protein was precipitated with 100 μl ice-cold 100% trichloroacetic acid. After centrifugation at 0° C, the supernatant was removed, stored at −20° C and assayed within 1 week. After thawing, the residual protein precipitate was removed from the supernatant solutions by extraction with five rinses of 1 ml diethyl ether. Residual traces of ether were removed by evaporation. The reaction was started by the addition of glutathione reductase, and the linear increase in absorbance was compared with that obtained using four standard solutions containing defined concentrations of reduced GSH. Results were expressed in nanomoles of GSH/10^5 cells.

Statistics. Statistical significance was determined using Student's *t*-test (unpaired). Values of $P < 0.05$ were considered to be significant.

Results

Chemosensitivity studies

The cytotoxicity of cisplatin and tetraplatin was evaluated using the MTT assay. Curves of the response to cisplatin by sensitive EMT6/P and resistant EMT6/CPR cells are shown in Fig. 1. In three repeated experiments, we found the mean ID_{50} value for EMT6/P cells to be 0.53 ± 0.17 cisplatin μM and that for EMT6/CPR to be 1.37 ± 0.2 μM. The resistance factor, i.e. the ratio of ID_{50} (EMT6/CPR): ID_{50} (EMT6/P), amounted to 2.56 ± 0.21 ($n = 3$). Curves of the response to tetraplatin by EMT6/P and EMT6/CPR cells are also shown in Fig. 1. The mean ID_{50} value for both cell lines was 0.38 ± 0.09 μM tetraplatin and the resistance factor was 1 ± 0.13 ($n = 3$).

Measurement of cellular GSH levels in EMT6/P and EMT6/CPR cells after exposure to cisplatin

In initial experiments, we compared the cellular GSH content of the parent cell line EMT6/P with that of the cisplatin-resistant subline EMT6/CPR. We observed that the mean cellular GSH content was dependent on the growth phase of the cells. In cells in the logarithmic phase, the mean GSH content in EMT6/P cells was 0.81 ± 0.11 nmol/10^5 cells ($n = 6$) and that in EMT6/CPR cells was 1.38 ± 0.14 nmol/10^5 cells ($n = 6$). Cell cultures in the plateau phase exhibited 50% lower values (EMT6/P: 0.43 ± 0.12 nmol/10^5 cells, $n = 3$; EMT6/CPR: 0.76 ± 0.13 nmol/10^5 cells, $n = 3$). The GSH levels in cisplatin-resistant EMT6/CPR cells were therefore 1.7-fold those in the sensitive parent cell line in both growth phases. Subsequent experiments were carried out using cells in the logarithmic growth phase.

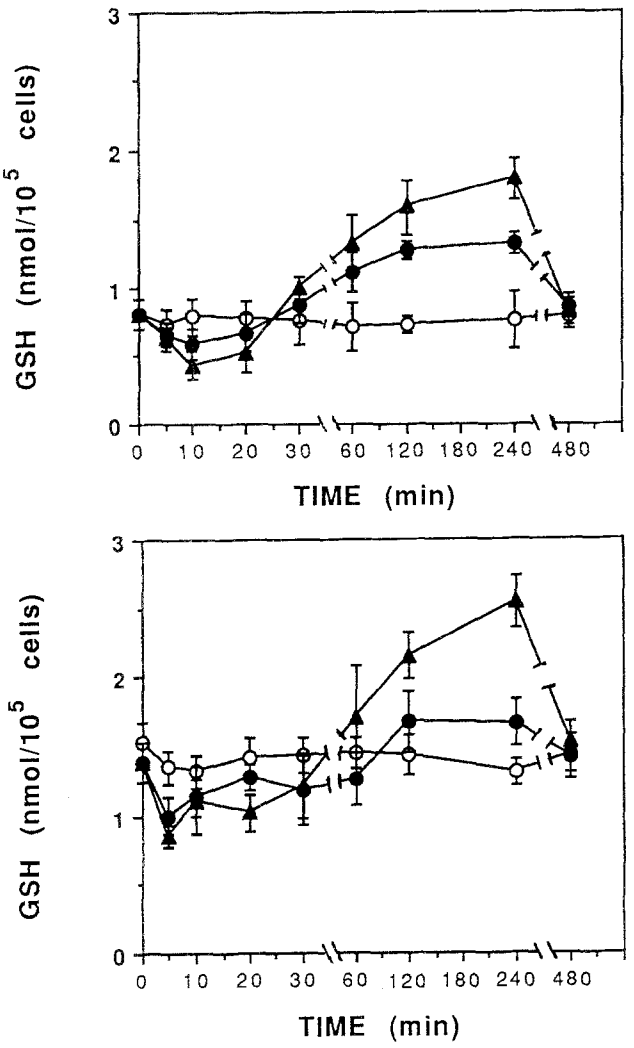

Fig. 2. Cellular GSH content of EMT6/P cells (*upper panel*) and EMT6/CPR cells (*lower panel*) incubated in the absence (○) or presence of 0.33 μM (●) and 3.33 μM (▲) cisplatin for various periods. Each point represents the mean value ± SD ($n = 4$)

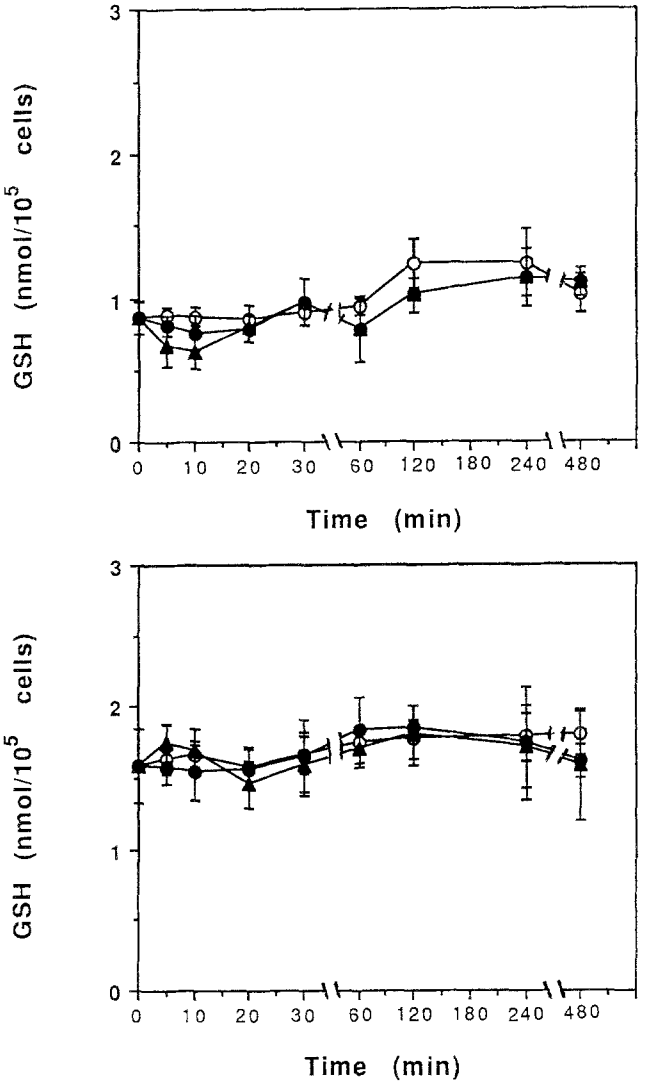

Fig. 3. Cellular GSH content of EMT6/P cells (*upper panel*) and EMT6/CPR cells (*lower panel*) incubated in the absence (○) or presence of 0.33 μM (●) and 3.33 μM (▲) tetraplatin for various periods. Each point represents the mean value ± SD ($n = 4$)

Results of GSH measurements in EMT6/P and EMT6/CPR cells after continuous exposure to 0.33 and 3.33 μM cisplatin are shown in Fig. 2. Within the 1st h, GSH levels dropped in both cell lines, the drop being more pronounced at the higher dose of cisplatin. The levels in EMT6/P cells were 50% lower than the control value after 10 min exposure to 3.33 μM cisplatin ($P < 0.01$). GSH levels in EMT6/CPR cells decreased by 30% after 20 min exposure to 3.33 μM cisplatin ($P < 0.05$). After 30 min incubation, the GSH content increased in both cell lines. The peak was reached after 4 h exposure to 3.33 μM cisplatin; in EMT6/P cells, GSH levels were 2.2-fold those in controls and in EMT6/CPR cells they were 1.8 times higher than control values. After 4 h exposure to 0.33 μM cisplatin, GSH levels showed a 1.7-fold elevation in the parent cell line and a 1.2-fold increase in the resistant subline. We could not observe any difference between the levels in controls and those in drug-exposed cells following 8 h incubation.

Measurement of cellular GSH in cisplatin-sensitive and -resistant cells after exposure to tetraplatin

Experiments using tetraplatin revealed no statistically significant change in cellular GSH content (Fig. 3). At no time point or concentration could we observe a difference in the GSH levels between cisplatin-sensitive and -resistant cells incubated in the presence or absence of tetraplatin.

Discussion

Elevated GSH levels in drug-resistant cells were first described nearly 30 years ago [11], and the relationship between intracellular GSH content and the cellular response to anti-neoplastic agents has been of interest since then. However, many questions remain open regarding the precise role of GSH in the development of drug resistance. As the major intracellular nonprotein sulfhydryl, GSH reacts

readily with electrophilic substances either directly or via a glutathione-*S*-transferase-mediated reaction [4]. Cisplatin has been shown to be sufficiently electrophilic to react with GSH directly [7]. Cell lines resistant to cisplatin often, albeit not always, display elevated GSH levels [2, 3, 16]. Also, depletion of GSH using L-buthionine-S-R-sulfoximine (BSO) has been shown to increase the sensitivity of resistant cells [9].

We derived a cisplatin-resistant variant of the mouse mammary tumour EMT6/P by stepwise in vitro exposure to increasing concentrations of cisplatin. This subline shows a 2.5-fold resistance to cisplatin as compared with the sensitive parent cell line EMT6/P. The development of drug resistance is accompanied by a 1.7-fold increase in cellular GSH. Interestingly, after 4 weeks' growth in the absence of cisplatin, the resistant cell line loses both its resistance phenotype and the elevated GSH levels (data not shown).

It should be emphasised that detoxification of substances involving GSH is a complex and dynamic process that concerns the activity of several related enzymes, the cellular GSH pool size, degradation of the adduct complex and recycling of the amino acids from GSH [4]. The kinetics of GSH changes after drug exposure should therefore give a more appropriate answer than the measurement of basal GSH levels. Recently, Meijer et al. [14] reported differences in GSH metabolism between cisplatin-sensitive and -resistant human lung cancer cell lines following exposure to the drug. These authors obtained a constant increase in the GSH content of the sensitive cell line and only slight changes in the levels in the resistant cell line.

In the present experiments, we observed a different pattern of changes in GSH levels. After exposure to cisplatin, the cellular GSH content dropped within the 1st h and subsequently increased in both the sensitive and the resistant cell line. The pattern of GSH changes was similar in both cell lines, although the cisplatin-resistant subline displayed higher GSH levels at all times. If cisplatin is inactivated by direct binding to the sulphydryl moiety of GSH, elevated levels could indeed protect the critical target DNA. Also, at the ID_{50} dose of cisplatin, the changes in GSH levels were less pronounced in the resistant cell line than in the parent line. This could indicate a more stable steady state of the GSH defence system in the resistant cell line at a critical dose of cisplatin.

One strategy for overcoming cisplatin resistance is the development of platinum analogues that retain activity in resistant cells [5]. Our cytotoxicity studies using the MTT assay showed that the cisplatin-resistant EMT6/CPR cells do not show cross-resistance to the 1,2-diaminocyclohexane platinum(IV) derivative tetraplatin. This observation is in agreement with other reports that tetraplatin may circumvent cisplatin resistance in some cell lines [1, 10]. The mechanisms underlying the observed difference between the cytotoxicity of cisplatin and that of tetraplatin are unclear at present [8, 18]. It may be that the selective activity of tetraplatin is attributable to the circumvention of detoxification pathways such as GSH metabolism.

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