O'-alkylguanine-DNA-alkyltransferase activity and nitrosoare sensitivity in human cancer cell lines

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Summary The DNA repair enzyme, O'-alkylguanine-DNA-alkyltransferase (ATase), is thought to be the principal mechanism conferring resistance to nitrosoare and related alkylating agents. We compared the activities of five human testis and five bladder tumour cell lines to two nitrosoares (N-nitroso-N-methylurea (MNU) and mitozolomide) with cellular levels of ATase. Enzyme levels ranged from 3 to 206 fmol mg-1 protein (0.1 x 106 to 5.1 x 108 molecules/cell) in the testis lines and from 11 to 603 fmol mg-1 (0.4 x 106 to 9.1 x 108 molecules/cell) in the bladder lines. Based on IC50s in an MTT assay, the testis tumour cell lines were, on average, four times more sensitive to MNU and six times more sensitive to mitozolomide than the bladder cell lines. The cytotoxicities of MNU and mitozolomide were closely related (R = 0.9). In the testis cell lines ATase activity (molecules/cell) was related to IC50s for mitozolomide (R = 0.97) but not MNU (R = 0.78). In the bladder cell lines and overall, ATase activity correlated with cellular sensitivity to neither agent. Relatively high levels of expression occurred in cells expressing low levels of ATase, and amongst cell lines expressing high levels of ATase, large differences in IC50s were observed. These results support the suggestion that resistance to nitrosoares can be mediated by mechanisms other than ATase and that at relatively high levels of expression, ATase does not confer resistance in proportion to its activity.

Cells expressing ATase are commonly resistant to the cytotoxic effects of mono- and bifunctional methylating and chloroethoxylines and alkylating agents (Pegg, 1990), including the chloroethyl nitrosoureas (CNU) used as chemotherapeutic drugs. ATase is thought to reduce the cytotoxicity of these agents by transferring the chloroethyl group from the O' position of guanine to a cysteine residue in the active site of the enzyme before interstrand crosslinks can be formed (Kohn, 1977; Erickson et al., 1980a; 1980b). Each enzyme molecule can remove only one alkyl group, as the reaction is auto-inactivating (Pegg, 1990). This may be of clinical relevance as it has been suggested that tumours with low levels of this enzyme are more likely to respond to chemotherapy using nitrosoares.

There are three main lines of evidence indicating that ATase influences sensitivity to nitrosoares. Firstly, cultured cells expressing low levels of the enzyme can be hypersensitive to nitrosoares (Day et al., 1980; Bodell et al., 1986; Jelinek et al., 1988; Smith & Brent, 1989), though this is not always the case (Samson & Linn, 1987; Maynard et al., 1989). Secondly, pretreatment of cells with agents which deplete endogenous ATase, such as alkylating agents or O'-methylguanine as free base, increases sensitivity to subsequent exposure to alkylating agents (Gibson et al., 1986; Gerson et al., 1988). Thirdly, transfection of the E. coli ada gene, encoding ATase, into mammalian cells deficient in ATase, can increase their resistance to alkylating agents (Margison & O'Connor, 1997).

Some types of cancer are relatively sensitive to nitrosoares and other chemotherapeutic drugs. For example, metastatic testis tumours are cured in over 80% of patients using combination chemotherapy (Peckham, 1988). We have shown that testis tumour cell lines retain sensitivity to anticancer agents in vitro (Walker et al., 1987; Masters et al., 1990), reflecting the clinical response of these tumours. To determine whether the sensitivity of testis tumour cells to nitrosoares might be related to ATase, we compared ATase levels and cellular sensitivities to two nitrosoares, MNU and mitozolomide, in five human testis and five bladder cancer cell lines, and two cisplatin-resistant sublines (Walker et al., 1990).

These two compounds were chosen as models for all mono- and bifunctional nitrosoares. They were selected specifically because their mechanisms of action are well-characterised and their toxicity would be expected to be associated with ATase levels, if this enzyme controls sensitivity in these cells (Gibson et al., 1984; Stevens et al., 1984; Pegg, 1990).

Materials and methods

Measurement of cytotoxicity of methylating and chloroethoxyline agents

The cytotoxicities of MNU (Sigma Chemical Co. Ltd., Poole, UK) and mitozolomide (May & Baker, Dagenham, UK) against the bladder cell lines RT112, RT112-CP, RT4, HT1376, HT1197 and MGH-U1 and the testis cell lines SuSa, SuSa-CP, 1618K, Tera II, GH and GCT27, were compared using the MTT assay, as described (Walker et al., 1990), except that the concentration of MTT (Sigma) used was 4mg ml-1. Both drugs were dissolved in DMSO and stored at -20°C. For each experiment, an aliquot was thawed and rapidly diluted to the appropriate concentration in complete medium and immediately added to the cells. Toxicity of MNU was assessed using a range of 0.25-200 μg ml-1 and toxicity of mitozolomide was assessed using a range of 0.1-50 μg ml-1. Drug was added 24 h after the cells were plated, and left for a further 6 days. A minimum of three separate experiments were performed for each cell line and each drug. Dose responses were plotted and IC50 values (i.e. concentration of drug which reduces the absorbance to 50% of the control) were calculated by linear regression analysis of the linear part of the graph.
Measurement of ATase levels in testis and bladder cell lines

Exponentially-growing cells were harvested by incubation in a 0.05% trypsin (Difco, London, UK), 0.016% EDTA (BDH, Poole, UK) solution for 5 min at 37°C. Cells were resuspended in medium containing 5% serum and then washed twice in PBSA. Cells were counted using a haemocytometer, and pellets containing 2 x 10^7 cells stored in liquid nitrogen. The frozen cell pellets were thawed into 1 ml Buffer 1 (50 mM Tris, 1 mM EDTA, 3 mM dithiothreitol at pH 8.3) and stored on ice. Cells were sonicated twice for 10 s each, firstly with 12 µm and secondly with 18 µm peak-to-peak distance. The protease inhibitor phenylmethysulfonyl fluoride (PMSF) (0.01 volume of 8.7 mg ml^{-1} PMSF in ethanol) was added immediately after the second sonication. Sonicates were then centrifuged at 16,000 r.p.m. at 4°C for 10 min, and the supernatants kept at 0°C.

ATase assays were carried out essentially as described (Morten & Margison, 1988), except that the total incubation volume was 400 µl. Positive and negative controls were included: the positive control was a range of six volumes of a semi-purified extract of E. coli containing the plasmid p061 which carries the E. coli ogt ATase gene (Margison et al., 1985) and incubation was at 37°C for 2 h. BSA solution (final concentration 2 mg ml^{-1}) and perchloric acid (PCA) (final concentration 1 m) were added simultaneously to every tube to precipitate protein and DNA. The mixture was hydrolysed at 75°C for 45 min. Samples were centrifuged and the precipitate washed once with 1 M PCA. Pellets were resuspended in 300 µl of 0.01 M sodium hydroxide. Ecoscient A scintillation fluid (3 ml) (Mensura Technology Ltd., Wigan, UK) was added and the amount of radioactivity in the protein measured. Protein content of the cell extracts was estimated using the Bradford assay (Bradford, 1976). The volumes of extracts used were protein limiting in the ATase assay. Estimations were performed on a range of 3 volumes of each extract. The assay was repeated two or three times, using different batches of cells.

ATase levels were expressed as the number of molecules/cell using the formula:

\[ \text{fmol ATase ml/extract} = \frac{\text{number of cells/ml}}{\text{Avogadro’s number}} \]

and as fmol mg^{-1} protein. Correlation coefficients were calculated using the Oxstat program. ATase levels in molecules/cell were used for these calculations. Mean IC50s for testis and bladder cell lines were compared using the Mann-Whitney U test. ATase levels in the parent and resistant cell lines (SuSa, SuSa-CP, RT112, RT112-CP) were compared using the Student’s unpaired t-test, also using the Oxstat program.

Results

Sensitivities of the testis and bladder cell lines to MNU and mitozolomide (expressed as IC50s), and ATase levels (expressed as fmol ATase mg protein and molecules x 10^4/cell) are shown in Table I. The testis cell lines were always more sensitive to MNU and mitozolomide than the bladder cell lines. Comparing the mean IC50s, the testis cell lines were 4.1-fold more sensitive to MNU and 6.2-fold more sensitive to mitozolomide than the bladder cell lines. The means were significantly different (P<0.001) for both MNU and mitozolomide, using the Mann-Whitney U test, and there was no overlap in IC50s between the testis and bladder cell lines.

ATase activity in four out of five bladder cancer cell lines was higher than in any testis cell line, ranging from 279 to 603 fmol mg^{-1} protein. In contrast, one bladder cell line, HT1197, had an ATase level of 11 fmol mg^{-1}, which was lower than all the testis cell lines except GCT7 (3.3 fmol mg^{-1}). When ATase activities were calculated as molecules/cell, the same pattern was observed (see Table I).

The relationships between IC50s and ATase levels (molecules/cell) in the testis and bladder tumour cell lines are shown in Figure 1. 1618K has the highest ATase level of all the testis cell lines, and is the most resistant to MNU and mitozolomide. GCT7 has the lowest ATase level, and is the most sensitive to both agents. However, 1618K and Tera II have similar IC50s to MNU and mitozolomide, but 1618K has an ATase level of 206 fmol mg^{-1} protein (5.1 x 10^4 molecules/cell), compared with 39 fmol mg^{-1} (2.0 x 10^4 molecules/cell) in Tera II. 1618K and SuSa have the same ATase levels based on protein measurements, but a 1.8-fold difference based on content/cell (5.1 and 2.8 x 10^4), which correlates more closely with the difference in sensitivity to MNU (2.3-fold).

Comparing sensitivity and ATase levels between cell types, GCT7 (testis) and HT1197 (bladder) have similar ATase levels (3.3 and 11 fmol mg^{-1} protein or 0.1 and 0.4 x 10^4 molecules/cell), but GCT7 is 50-fold more sensitive to MNU, and 14-fold more sensitive to mitozolomide, than HT1197. RT4 (bladder) cells have only a 26% (based on protein) or 16% (based on content/cell) higher ATase level than SuSa and 1618K (testis) cells, but are five times more resistant to mitozolomide. Four of the testis cell lines have higher ATase levels than HT1197, but they are 2–6 times more sensitive to MNU and mitozolomide.

Comparing cisplatin-resistant SuSa-CP with its sensitive parental line, SuSa-CP has a significantly higher level of ATase than SuSa (470 versus 206 fmol mg^{-1} protein; 7.4 x 10^4 versus 2.8 x 10^4 molecules/cell) (P<0.01 in a Student’s unpaired t-test). There is also a difference in sensitivity:

| Table I | ATase activity and sensitivity to MNU and mitozolomide |
| --- | --- |
| **Cell line** | **fmol mg^{-1} protein** | **ATase level** | **molecules x 10^4/cell** | **MNU** | **IC50 ± s.e. (µg ml^{-1})** | **mitozolomide** | **IC50 ± s.e. (µg ml^{-1})** |
| **Testis:** | | | | | | | |
| SuSa | 259 | 174 | 186 | 206 | 2.56 | 3.73 | 1.99 | 2.76 | 12.2±3.4 | 1.34±0.18 |
| Tera II | 62 | 16 | 39 | 1.69 | 2.29 | 1.99 | 26.1±8.1 | 1.15±0.22 |
| GH | 121 | 91 | 106 | 1.54 | 1.51 | 1.52 | 31.3±1.3 | 0.68±0.16 |
| 1618K | 230 | 183 | 206 | 5.35 | 4.94 | 5.14 | 28.1±2.8 | 1.82±0.31 |
| GCT7 | 4.0 | 2.7 | 3.3 | 0.11 | 0.09 | 0.10 | 1.18±0.2 | 0.33±0.06 |
| SuSa-CP | 450 | 490 | 470 | 3.68 | 8.43 | 7.40 | 29.0±1.8 | 4.19±0.50 |
| **Bladder:** | | | | | | | |
| RT112 | 396 | 378 | 387 | 6.32 | 6.62 | 6.47 | 56.2±4.1 | 4.46±0.35 |
| RT4 | 527 | 280 | 279 | 6.20 | 5.60 | 5.93 | 66.7±2.0 | 8.84±0.44 |
| HT1376 | 520 | 378 | 419 | 9.09 | 5.72 | 10.23 | 65.6±3.1 | 9.94±1.88 |
| HT1197 | 20 | 1.5 | 10.7 | 0.74 | 0.96 | 0.42 | 59.6±4.0 | 4.49±0.49 |
| MGH-U1 | 718 | 534 | 558 | 603 | 8.43 | 8.79 | 10.24 | 9.15 | 53.9±4.9 | 5.27±0.50 |
| RT112-CP | 450 | 215 | 237 | 301 | 5.42 | 5.06 | 4.22 | 4.90 | 67.3±4.9 | 10.49±0.64 |

*Results from individual experiments are shown.*
comparing IC50s, SuSa-CP is 2.4-fold more resistant to MNU, and 3.1-fold more resistant to mitozolomide. RT112 and RT112-CP have similar ATase levels, and similar sensitivity to MNU. However, RT112-CP is 2.3-fold more resistant to mitozolomide than the parental line.

The rankings of the individual cell lines in their sensitivities to MNU and mitozolomide were similar. The only correlation between ATase levels (expressed as molecules \( \times 10^5 \) cell) and mitozolomide IC50s was within the testis cell line group, when considered alone. However, overall there was no correlation between MNU or mitozolomide sensitivity and ATase activity.

Discussion

We have shown that levels of ATase activity are not associated with either sensitivity or resistance to nitrosoureas in a group of 10 testis and bladder cancer cell lines. The testis cell lines were uniformly more sensitive to MNU and mitozolomide than the bladder lines, but this was not associated with major differences in ATase levels between the two groups. If the testis cells are considered in isolation, ATase levels correlated with sensitivity to mitozolomide but not MNU.

Low ATase activity is associated with sensitivity to CNUs in human and murine cell lines in some studies (Day et al., 1980; Bodell et al., 1986; Jelinek et al., 1988; Smith & Brent, 1989) but not others (Samson & Linn, 1987; Maynard et al., 1989). It is difficult to relate actual ATase levels to sensitivity or resistance, because the ATase levels associated with resistance to CNUs differ widely between studies. An ATase level as low as 33 fmol mg\(^{-1}\) protein correlated with resistance to the cytotoxic effects of MTIC in human melanoma cell lines (Maynard et al., 1989). Five melanoma cell lines with ATase levels of 13 fmol mg\(^{-1}\) or lower were 4 to 7-fold more sensitive to MTIC than four melanoma cell lines with ATase activity of at least 33 fmol mg\(^{-1}\) protein. A small difference in ATase activity was associated with a relatively large difference in sensitivity. In contrast, a human rhabdomyosarcoma cell line containing 3800 fmol ATase mg\(^{-1}\) protein was only 5–6 times more resistant to CNUs than a rhabdomyosarcoma cell line which lacked measureable ATase (Smith & Brent, 1989). In this case, a very large difference in ATase activity was associated with a relatively modest difference in CNU sensitivity. Thus it seems unlikely that the very small difference in ATase activity between RT4 (bladder) and SuSa or 1618K (testis) cell lines would alone account for the 5–6-fold difference in mitozolomide sensitivity in this study.

While in the testis cell lines there appears to be a reasonable correlation between ATase levels (expressed/cell) and the IC50s for mitozolomide, there are several possible mechanisms besides ATase level which could explain differential sensitivity to CNUs between bladder and testis lines and within the bladder lines. These include differences in uptake of drug and binding to intracellular molecules other than DNA, such as glutathione. Resistant cells may be better able to tolerate or bypass the O\(^6\)-alkylguanine lesions (Fox & Roberts, 1987). Repair mechanisms other than ATase capable of removing O\(^6\)-lesions exist. For example, V79 and V79/79 Chinese hamster cell lines, which lack ATase activity, can remove O\(^6\)-butylguanine from DNA (Boyle et al., 1987). This is probably by nucleotide excision repair, the process which recognises bulky adducts including uv-induced pyrimidine dimers. In the case of V79/79 cells it has been shown that O\(^6\)-methylguanine can also be removed from DNA, presumably by an excision repair process (Boyle et al., 1987). The close correlation between MNU and mitozolomide sensitivity in individual cell lines does suggest that the non-ATase resistance mechanism may be acting on damage produced by both agents and might thus be an analogous excision repair system.

Another factor that might contribute to differential sensitivity is the existence of mechanisms of cell killing by CNUs besides production of O\(^6\)-lesions. Alkylation agents produce twelve different lesions in DNA, in varying proportions, most nitrogens and oxygen atoms being possible sites of attack (Margison & O'Connor, 1989). Besides O\(^6\)-alkylguanine, other lesions produced in DNA include N\(^7\)-alkylguanine and N\(^3\)-alkyladenine. For methylating agents, N\(^3\)-methyladenine, but not O\(^6\)-methylguanine or N\(^7\)-methylguanine (the major product), blocks DNA synthesis (Margison & O'Connor, 1989).

ATase levels and MNU and mitozolomide sensitivities were measured in cisplatin-resistant sublines of SuSa and RT112. Cisplatin is believed to bind predominantly to the N\(^7\) position of guanine, and to form DNA inter- and intranucleoside crosslinks and DNA-protein crosslinks. No correlation has been found between ATase levels and the extent of cisplatin-induced DNA interstrand crosslinking in human tumour cell lines, suggesting that ATase is not involved in repair of cisplatin-induced lesions (Laurent et al., 1981; Gibson et al., 1985). However, there is evidence for ATase activity being increased following exposure of cells to cisplatin in vitro. For example, in the H4 rat hepatoma cell line ATase levels increased approximately 4-fold 48 h after a 1 h exposure to cisplatin (Lefebvre & Laval, 1986). In the present study, there was no evidence for increased ATase level in the cisplatin-resistant subline of RT112. MNU sensitivity was unchanged, but RT112-CP was 2.3-fold more resistant than RT112 to mitozolomide. This suggests that RT112-CP has acquired resistance to crosslinking agents, but not to functional alkylating agents. This result implies that the increased mitozolomide resistance of RT112-CP is mediated by a factor other than ATase level. On the other hand, the cisplatin-resistant testicular cell line, SuSa-CP, had a higher ATase level than the parental line, and was cross-resistant to both MNU and mitozolomide. In this case, the increased ATase which may be a consequence of exposure to cisplatin, may be contributing to MNU and mitozolomide resistance in SuSa-CP.

More effective therapy for incurable cancers may result from understanding how testis tumours are sensitive to anticancer agents. We have explored some of the possible mechanisms using the model system described in this study.

For example, sensitivity of testis cancers cannot be explained by cell kinetics (Walker et al., 1987; Walker, 1990), spontaneous or induced mutation frequencies (Parris et al., 1990), and failure to inhibit DNA synthesis (Parris et al., 1988), glutathione-S-transferase or topoisomerase II levels (Fry et al., 1991), level of drug binding to DNA (Walker, 1990) or levels of DNA inter- or intrastrand crosslink repair (Bedford et al., 1988). The data presented here indicate that ATase levels are not an exclusive factor in resistance or sensitivity to mitozolomide in these cell lines.

Future studies will focus on fundamental aspects of DNA repair and the signalling pathways that determine whether a cell survives or dies following drug-induced damage.
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