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Original Paper

The Antitumour Activity of Alkylating Agents is not Correlated with the Levels of Glutathione, Glutathione Transferase and O^6-Alkylguanine-DNA-alkyltransferase of Human Tumour Xenografts

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Twenty-three human xenografts, including five colon, five gastric, nine lung (three small cell lung cancer) and four breast carcinomas, were investigated for their sensitivity to nitrosoureas, dacarbazine (DTIC), cyclophosphamide (CTX) and cisplatin (DDP). In 12 cases, at least one of the drugs produced complete or partial remission, in 2, a minor regression was observed and in the other 9, treatment was ineffective. The level of sensitivity to each drug, using a score from 1 to 5, was correlated to three biochemical parameters reported to be involved in resistance to alkylating agents: glutathione (GSH), glutathione transferase (GST) and O^6-alkylguanine-DNA-alkyltransferase (AGT). A wide variability was found in these parameters in the xenografts investigated. No correlation was found between any of the three parameters and sensitivity to the drugs used or between sensitivity to one drug and to any of the other drugs tested. These results illustrate the complexity of the question of resistance to alkylating agents and indicate that, at least in xenografts, the biochemical parameters examined are not predictive of response to alkylating agents. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: DNA repair, glutathione, O^6-alkylguanine-DNA-alkyltransferase, human tumour, xenografts

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INTRODUCTION

THE CYTOTOXIC activity of methylating and chloroethylating agents depends closely on intracellular levels of the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (AGT) [1–3]. It has been proposed that in a cell deficient in AGT, alkylation of O^6-guanine caused by chloroethylnitrosoureas is followed by a re-arrangement and subsequent reaction leading to a DNA interstrand crosslink between a guanine and a cytosine on the opposite strand [4–6]. There appears to be a good correlation between the deficiency in AGT, the amount of chloroethylnitrosourea-induced DNA interstrand crosslinks and sensitivity to the cytotoxicity of these drugs [7–10]. In almost all studies, cell lines growing in culture have been exposed to nitrosoureas, but there are very limited data obtained *in vivo* supporting the view that AGT levels are important for the antitumour activity of nitrosoureas against human tumour xenografts [11–13]. Intracellular AGT levels appear unimportant for the cytotoxicity of other alkylating agents such as nitrogen mustards or cisplatin (DDP) [14] and this has been explained by the fact that these drugs cause negligible levels of adducts at the guanine O^6 position. For all alkylating agents, a major mechanism of resistance appears to be related to the glutathione (GSH) levels and glutathione transferase (GST), although results are still conflicting [15, 16].

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In the present study, we used a large panel of human xenografts to assess the relationship between *in vivo* sensitivity to nitrosoureas, cyclophosphamide (CTX) and DDP and the tumour levels of AGT, GSH and GST.

MATERIALS AND METHODS

Nude mice and tumour

Human tumours established in serial passages in nude mice (NMRI genetic background) were used [17, 18]. Tumours originated from colon (CXF), stomach (GXF), lung (LXF) and mammary gland (MAXF). The animals were housed in Makrolon cages set in laminar flow racks. A total of 23 xenografts were employed. The histological appearance of the murine tumours was strikingly similar to the original human tumour in most cases and only 20% of the xenografts showed minor variations in their degree of differentiation. The human origin of the tumours was ascertained by isoenzymatic and immunohistochemical methods [19]. Tumours were implanted subcutaneously in both flanks of nude mice. Five to six nude mice bearing tumours were used for the control or the treated group. Treatment was initiated when the mean tumour diameters were 6–7 mm.

Drugs

All compounds were administered following a clinical-type schedule at their maximum tolerated doses (MTD), defined as up to an LD_{20} in tumour bearing mice 2 weeks after the last treatment. Xenografts have demonstrated high predictivity in comparison with the tumour response in patients [20–22]. The MTD of the nitrosourea CCNU (cyclohexyl-chloroethylnitrosourea) and HECNU (hydroxyethyl-chloroethylnitrosourea) were 20 mg/kg and 10–12 mg/kg in a single-dose schedule. CTX and DDP had an MTD of 200 and 6.4 mg/kg/day, given on days 1 and 15. CCNU, HECNU and CTX were injected intraperitoneally, DDP was administered subcutaneously.

The evaluation of drug sensitivity was performed between the fifth and 20th passage. In order to illustrate the degree of reproducibility of the antitumour activity, the results obtained in single experiments are shown in Table 1.

GSH, GST and AGT were assayed in tumour specimens snap-frozen in liquid nitrogen and stored at $-80°C$.

Drug effectiveness

Treatment was started as soon as the tumours reached a median diameter of 6 mm, depending on the doubling time between days 12 and 42. Mice were randomly assigned to treatment groups or the control group (five to six mice per group). The tumour size was calculated according to the formula length×width, using two perpendicular tumour diameters measured with calipers. The antitumour effect was evaluated by following maximal tumour regression or after

Table 1. *Effect of different alkylating agents on relative tumour size in human xenografts**

	Nitrosoureas		DTIC		CTX		DDP	
Tumour	Values	Mean	Values	Mean	Values	Mean	Values	Mean
Colon								
CXF 158	27, 43, 36	35 ± 8	26, 45	36 ± 10	280, 166	223 ± 57	207, 171	189 ± 18
CXF 280	44, 37, 47, 35, 50	43 ± 6	44		39		68, 54	71 ± 3
CXF 886			207		105		217, 163	190 ± 27
CXF 975			309		319		372	
CXF 1103	364		577		353		344, 868	606 ± 262
Gastric								
GXF 97	26, 19, 38, 27	28 ± 8	403		66, 70	68 ± 2	11	
GXF 209	80, 92	86 ± 6	189		132		42	
GXF 251	302, 383	343 ± 41	296		254		173, 218, 201, 272	216 ± 42
GXF 281	38, 34, 40, 37	37 ± 3	118		145		40	
GXF 324			269		227		256, 197	227 ± 30
Lung, adenocarcinoma								
LXFA 526			220		164		199	
LXFA 629	215, 190	203 ± 13			124		180, 347, 295	274 ± 84
Lung epitheloid								
LXFE 211	76, 86, 98	87 ± 11			193, 228	201 ± 28	57	
LXFE 397	22		24		627, 387	507 ± 120	193	
LXFE 409	400, 285, 338	341 ± 58	265		51		50	
Lung, large cell								
LXFL 529	425, 435	430 ± 5	51, 57	54 ± 3	5, 1, 0, 22	7 ± 10	63, 65, 86, 79	73 ± 11
Lung small cell								
LXFS 538	19, 21	20 ± 1	87		11, 8	10 ± 2	90, 125	108 ± 18
LXFS 573					65, 123	94 ± 30	255	
LXFS 650	437		389		81, 116, 98	98 ± 18	207	
Mammary								
MAXF 401	13, 10, 18, 19	15 ± 4	21		18, 22	20 ± 2	16	
MAXF 449	40, 35	38 ± 3	173, 100	137 ± 37	48, 44, 37, 15	36 ± 15	25, 27, 67, 56	44 ± 21
MAXF 583	138				97		37	
MAXF 1322	60, 61	60 ± 1			36			

*Comparison of relative tumour volume (%) on day *x* versus day 0 (100%). Day *x* was after 3–4 weeks in progressive tumours and after maximal regression in regressive tumours. Whenever possible, the mean ± standard deviation is shown. DTIC, dacarbazine; CTX, cyclophosphamide; DDP, cisplatin.

3–4 weeks in non-regressing tumours. Relative tumour sizes (RTS) were calculated for each single tumour by dividing the tumour size on day x by the tumour size on day 0 at the time of randomisation. Median RTS were used for further evaluation. The effect of treatment was classified as in clinical studies as complete remission (RTS $\leq$ 20% of initial value), partial remission (11–50%), minor regression (51–75%), no change after day 21 or day 28 (76–124%) or progression ($\geq$ 125%).

AGT activity

AGT activity was assayed following the procedure previously described [23, 24]. Briefly, the tumour samples were sonicated in 50 mM Tris–HCl, pH 8.3, 1 mM ethylenediamine tetra-acetic acid (EDTA), 3 mM dithiothreitol then phenylmethylsulphonyl fluoride was immediately added. The sonicates were then centrifuged and increasing amounts of the supernatants were incubated with [methyl-^{3}H]DNA. The AGT content was determined by liquid scintillation counting of protein precipitates. The results were expressed as fmol methyl transferred per mg of protein content in the sample assayed.

GST and GSH activities

Tissue samples were washed with cold phosphate buffered saline (PBS), minced with scissors and sonicated in PBS at 4°C for 10 sec followed by a 10 sec pause. The sonication schedule was repeated three times, the homogenate was then centrifuged at 10 000***g*** at 4°C for 10 min and the supernatant analysed for GST activity, GSH and protein content. GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to Habig and Jakoby [25]. The method of Tietze [26] was used for total GSH which was measured after protein denaturation with sulphosalicylic acid. Briefly, one volume of 0.55 M sulphosalicylic acid was added to three volumes of supernatant and kept in ice for 1–4 h to allow precipitation of proteins. The mixture was centrifuged at 10 000***g*** for 10 min at 4°C and the supernatant analysed for total GSH. The protein concentration was measured using the Bio-Rad, (Milan, Italy) protein assay standard procedure.

RESULTS

Table 2 shows the GSH, GST and AGT levels in 23 human xenografts, the majority of which are characterised for their *in vivo* sensitivity to nitrosoureas, DTIC, CTX and DDP. There was a high degree of heterogeneity for all parameters. GSH, GST and AGT per mg of protein ranged between 2.3 and 91.2 nmol, 103 and 862 U and 5 and 3,717 fmol, respectively. Although the numbers of each tumour type were too small to permit any definitive conclusions, GSH levels appeared to be lower in colon carcinoma xenografts than in the other tumours. When the sensitivity to

Table 2. Sensitivity of human xenografts to different alkylating agents, and tumour levels of glutathione (GSH), glutathione transferase (GST) and O^6-alkylguanine-DNA-alkyltransferase (AGT)

	Drug sensitivity				GSH, GST, and AGT levels		
Tumour	Nitrosoureas	DTIC	CTX	DDP	GSH ± S.D.*	GST ± S.D.†	AGT ± S.D.‡
Colon							
CXF 158	4	4	1	1	3.0 ± 2.2	298 ± 83	31 ± 3
CXF 280	4	4	4	3	6.7 ± 0.0	103 ± 15	25 ± 4
CXF 886		1	2	1	2.3 ± 0.7	329 ± 94	1494 ± 1
CXF 975		1	1	1	5.5 ± 4.3	411 ± 235	947 ± 37
CXF 1103	1	1	1	1	2.4 ± 1.0	335 ± 50	14 ± 3
Gastric							
GXF 97	4	1	3	5	35.7 ± 0.7	678 ± 128	2810 ± 401
GXF 209	2	1	1	4	17.5 ± 4.2	699 ± 105	261 ± 22
GXF 251	1	1	1	1	47.6 ± 8.6	617 ± 118	1418 ± 32
GXF 281	4	2	1	4	2.8	388 ± 24	14 ± 0
GXF 324		1	1	1	91.2 ± 9.3	449 ± 235	645 ± 3
Lung, adenocarcinoma							
LXFA 526		1	1	1	23.3 ± 1.8	862 ± 81	272 ± 43
LXFA 629	1		2	1	87.8 ± 55.3	584 ± 103	1584 ± 177
Lung, epitheloid							
LXFE 211	2		1	3	78.8 ± 8.2	677 ± 102	305 ± 65
LXFE 397	4	4	1	1	18.4 ± 3.5	263 ± 53	5 ± 0
LXFE 409	1	1	3	3	28.2 ± 4.6	251 ± 23	1833 ± 0
Lung, large cell							
LXFL 529	1	3	5	3	24 ± 10.3	142 ± 44	639 ± 23
Lung, small cell							
LXFS 538	4	2	5	2	34.7	200 ± 22	1394 ± 35
LXFS 573			2	1	54.6 ± 4.8	600 ± 42	1397 ± 79
LXFS 650	1	1	2	1	43.4 ± 12.9	126 ± 2	917 ± 46
Mammary							
MAXF 401	5	4	4	5	38.1 ± 6.5	325 ± 31	477 ± 59
MAXF 449	4	1	4	4	36.7 ± 14.8	374 ± 26	3717 ± 313
MAXF 583	1		2	4	2.5 ± 0.7	218 ± 52	1787 ± 18
MAXF 1322	3		4		46.6 ± 14.1	214 ± 68	742 ± 101

1, progression (> 125% of initial tumour volume); 2, no change (75–125%); 3, minor regression (50–75%); 4, partial remission (20–50%); 5, complete remission (< 20%). *nmol GSH/mg protein; †units GST/mg protein; ‡fmol AGT/mg protein. DTIC, dacarbazine; CTX, cyclophosphamide; DDP, cisplatin; S.D., standard deviation.

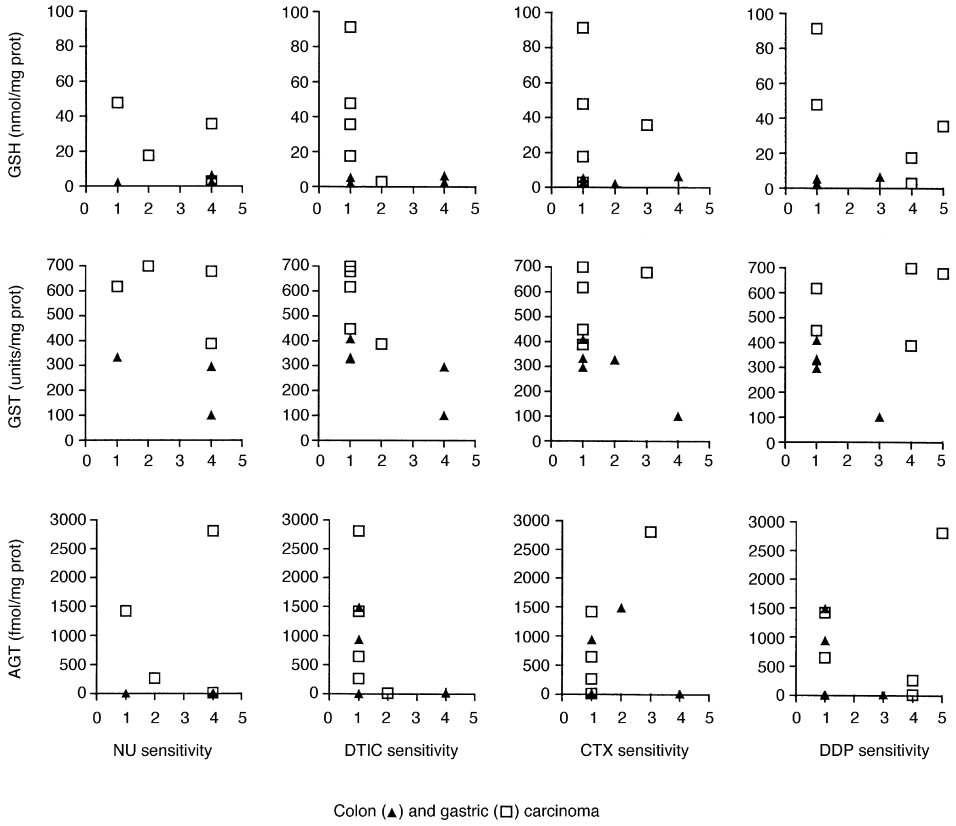

Figure 1. Sensitivity to nitrosoureas (NU), dacarbazine (DTIC), cyclophosphamide (CTX) or cisplatin (DDP) in relation to the levels of glutathione (GSH) and the activity of glutathione transferase (GST) and O^6-alkylguanine-DNA-alkyltransferase (AGT) in colon and gastric carcinoma xenografts.

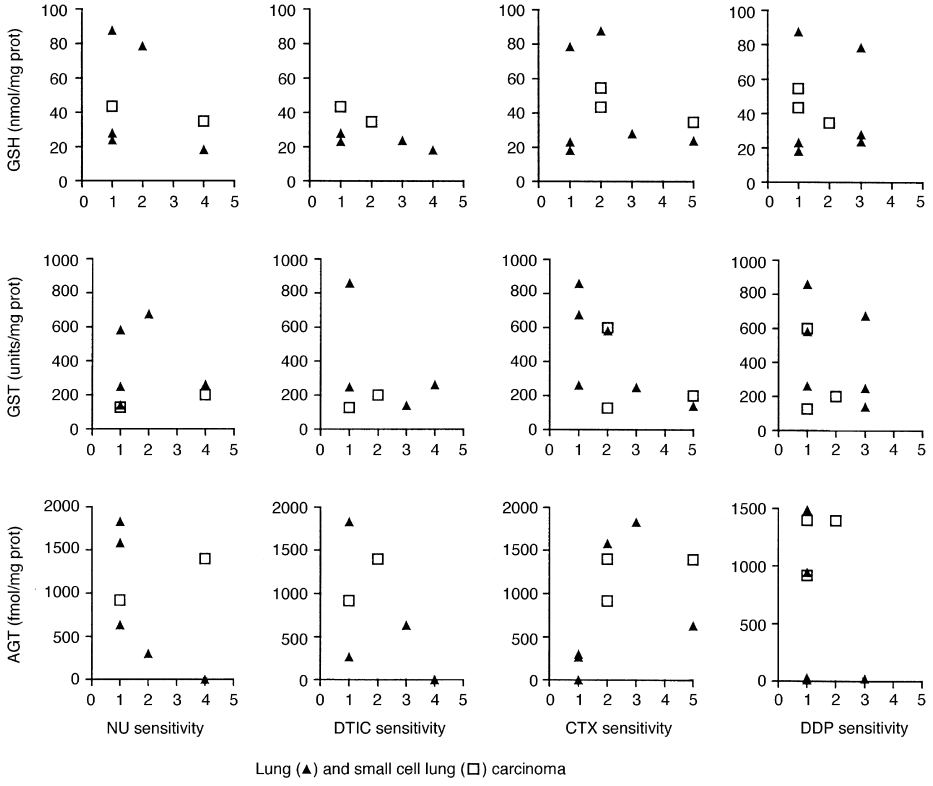

Figure 2. Sensitivity to nitrosoureas (NU), dacarbazine (DTIC), cyclophosphamide (CTX) or cisplatin (DDP) in relation to the levels of glutathione (GSH) and the activity of glutathione transferase (GST) and O^6-alkylguanine DNA-alkyltransferase (AGT) in lung carcinoma xenografts.

nitrosoureas, DTIC, CTX or DDP of all these tumours was plotted against the levels of GSH, GST and AGT, no statistical correlation was found. These data are illustrated for each tumour type in Figures 1–3. No correlation was found between drug sensitivity and the biochemical parameters investigated.

Figure 4 shows that there was also no correlation between sensitivity to one drug and sensitivity to any one of the other three drugs, supporting the view that the determinants of sensitivity differ for these four drugs.

DISCUSSION

The present study found no correlation between sensitivity to nitrosoureas, DTIC, CTX or DDP and the tumour level of GSH and GST and AGT. GSH and GST have been reported to be involved in the cellular detoxification of alkylating

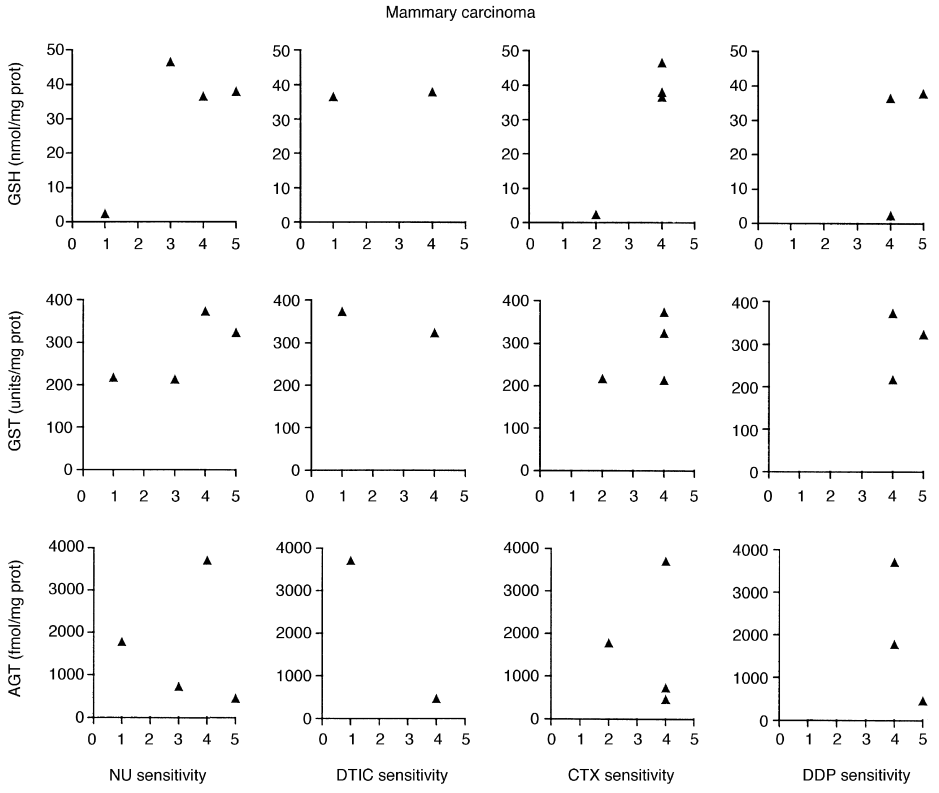

Figure 3. Sensitivity to nitrosoureas (NU), dacarbazine (DTIC), cyclophosphamide (CTX) or cisplatin (DDP) in relation to the levels of glutathione (GSH) and the activity of glutathione transferase (GST) and O^6-alkylguanine-DNA-alkyltransferase (AGT) in mammary carcinoma xenografts.

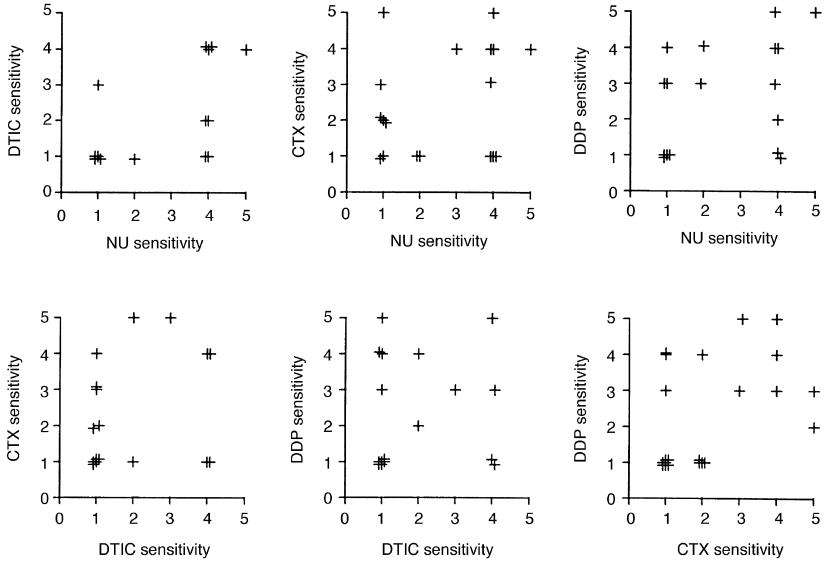

Figure 4. Sensitivity to one drug in relation to the sensitivity to each of the other three nitrosoureas (NU), dacarbazine (DTIC), cyclophosphamide (CTX) and cisplatin (DDP).

agents and, in some cases, resistance to these drugs appears to be related to the levels of GSH or to the activity of GST [15, 16]. However, the importance of GSH levels and GST activity is still much debated. Tumours treated with alkylating agents and DDP seem to show higher levels of GSH and GST, but this does not necessarily imply that GSH and GST are involved in the resistance mechanisms [27]. Recent studies in which cells were transfected with GST isoform genes failed to find any correlation between GST expression and the resistance to alkylating agents or DDP [28, 29]. Therefore, the present finding that GSH tumour content and GST tumour activity are not related to the sensitivity to nitrosoureas, DTIC, CTX and DDP is not surprising.

The lack of correlation between CTX and MP sensitivity and AGT levels could also be anticipated on the basis of studies in cancer cell lines growing *in vitro* [30]. What was not expected was the lack of correlation between nitrosoureas or DTIC sensitivity and AGT levels. The importance of AGT in resistance to nitrosoureas or methyltriazenes has in fact been highlighted in a number of previous reports [1, 2, 4, 5, 31, 32]. In addition, it has been demonstrated that a tumour cell which does not express the AGT gene and is very sensitive to nitrosoureas or methylating agents can become resistant to these drugs when transfected with the bacterial or mammalian gene encoding for this protein [33–35].

The majority of published studies have been conducted using cultured cells and not *in vivo* growing tumours. The present study is one of the few correlating the levels of AGT to the *in vivo* sensitivity to nitrosoureas or methyltriazenes. Therefore, the differences may be due to factors other than cellular sensitivity. For example, a tumour may have a low AGT content and, thus, be potentially sensitive, but the drug does not achieve high enough levels to exert its antitumour activity. However, since the *in vivo* treatment was started very soon after tumour transplant, this is unlikely. Another point is that the same tumour may be heterogeneous, containing cellular populations with different degrees of sensitivity to nitrosoureas or methylating agents. AGT levels were measured in tumour biopsies and may be averages; resistant clones may account for only a small fraction of the tumour, but are nevertheless relevant when antitumour activity is assessed.

However, correlations between the sensitivity to chloroethylating and methylating agents of cell lines growing *in vitro* and their AGT content have not been found in all studies. Walker and colleagues found no *in vitro* cell sensitivity and AGT content [36], and some have pointed out that apart from AGT, other mechanisms might affect the degree of cytotoxicity of methylnitrosourea and methylating agents [37–39]. Lefebvre and Laval investigated two cell lines that differed in their sensitivity to these compounds but had similar levels of AGT, and suggested that other DNA lesions might be responsible for the difference in cytotoxicity [40].

Cell lines resistant to methylating agents but with low levels of AGT have been reported to have mutations of proteins involved in mismatch repair [41, 42]. These cell lines, tolerant to methylating agents, were not resistant to nitrosoureas, implying that mismatch repair is not essential for the nitrosourea-induced DNA damage. Other mechanisms of DNA repair, such as N3 methyladenine glycosylase or excision repair mechanisms, may be involved [43, 44].

The finding that tumours with high levels of AGT were sensitive to nitrosoureas (e.g. mammary MAXF 449, lung S LXFS538, gastric GXF 97) implies that the alkylation of O^6 guanine is not the crucial lesion in all cases and other lesions may be involved.

Apart from specific lesions and factors involved in the mode of action of alkylating agents, there is growing evidence that other events downstream to the drug's effect on DNA (e.g. cell cycle checkpoints, cell death mechanism) play an important part in the ultimate cytotoxic effect of DNA damaging agents [45, 46].

In conclusion, the present study confirms that the determinants of sensitivity and resistance to alkylating agents are still only partially understood. The findings in human xenografts do not support the theory that by measuring AGT, GSH and GST in tumour biopsies of cancer patients it should be possible to select those cases that will respond to therapy with alkylating agents.

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