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Effect of various chemosensitizers on chemoresistance to adriamycin in MIP-101 cell line, a colon carcinoma cell line: analysis of glutathione and related enzymes

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We evaluated the multidrug resistance modulating effect of verapamil, buthionine sulfoximine, trifluoperazine and tamoxifen in a human colorectal cell line resistant *de novo* to adriamycin. We studied the effect of these chemosensitizers on glutathione content, glutathione reductase, transferases, peroxidases, γ-glutamyl transpeptidase and glucose-6-phosphate dehydrogenase activities. The ratio of the activities between resistant and sensitive cells treated by these compounds as compared with the ratio of the untreated cells decreases, thus contributing to the reversal of chemoresistance. This study implies a role of glutathione and related enzymes in chemoresistance to adriamycin, although this is certainly not the sole mechanism.

Key words: Adriamycin, chemoresistance, glutathione, MIP-101, reversion.

Introduction

Multidrug resistance (MDR), characterized by resistance to structurally unrelated drugs, represents a major clinical problem in cancer therapy. Such a resistance can be an intrinsic property of a tumor or can be acquired during apparently successful courses of chemotherapy. Multiple mechanisms of resistance possibly account for the complex patterns of drug resistance.[1–3]

The major mechanism described for pleiotropic drug resistance is an increased expression of an 170 kDa membrane glycoprotein (P-glycoprotein) which is believed to act as a drug efflux 'pump'.[4–6] However, alternative mechanisms of resistance to anti-cancer agents have been described, including alterations of topoisomerase II activity and modifications in the glutathione (GSH) metabolizing system.[1,7,8].

Anti-cancer agents such as intercalators, e.g. adriamycin and epipodophyllotoxins, are thought to act via interaction with topoisomerase II.[9,10] This nuclear enzyme, essential for a number of vital processes (i.e. replication), has been associated with the development of resistance in some instances.[8,11]

A potential mechanism of resistance to adriamycin is an increase in the GSH metabolizing system. Increases in GSH and alterations in the enzymes involved in glutathione synthesis, metabolism and utilization are implied in resistance in some instances.[12,13] High concentrations of GSH can modulate the activity of many chemotherapeutic agents, including adriamycin, by direct chemical reaction with the drug or a reactive species generated during drug activation.[14] Indeed, GSH is a substrate for several phase II detoxifying enzymes, including the selenium-dependent GSH peroxidase and GSH transferase families. In particular, glutathione S-transferase (GST) has been implicated. Soluble human GSTs have been classified into four classes: α, μ, π and θ, on the basis of structural, immunological and enzymatic properties.[15–17] The transfection of genes encoding for human π and α class GSTs into yeast cells has been shown to confer resistance to adriamycin.[18] Otherwise, glutathione peroxidases can reduce hydrogen peroxide and organic peroxides. Increases in the activity of detoxifying enzymes can render tumor cells less susceptible to damage by adriamycin.[19]

Thus, one of the more challenging problems in cancer research is understanding how tumor cells become resistant to cancer chemotherapeutic agents. After this, rational strategies to exploit or circumvent this resistance would rapidly follow. The chemosensitizers belong to a variety of

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structural classes, although a high hydrophobicity and an ability to diffuse through the cell membrane seem to be common requirements.[20]

In the present study, we have used a MIP-101 human colorectal cell line resistant to adriamycin. This *de novo* resistant cell line is characterized by increased intracellular GSH content, elevated GSH peroxidase activity and diminished GSH transferase activity.[12] We have investigated the relative roles that GSH metabolism and related enzymes play in this cell line in chemoresistance by using various known chemosensitizers. Among the chemosensitizing agents studied were verapamil (verap; a calcium channel blocker), buthionine sulfoximine (BSO; a selective inhibitor of glutathione synthesis), trifluoperazine (trifluo; a calmodulin inhibitor) and tamoxifen (tamox; an antiestrogen). The parameters studied were the expression of P-glycoprotein, the content of GSH, the activity of GSH transferases, peroxidases (characteristics developed by Kramer in DLD-1 and MIP-101 untreated cells), reductase, γ-glutamyl-transpeptidase (enzymes involved in GSH metabolism) and glucose-6-phosphate dehydrogenase (enzyme implicated in the hexose monophosphate pathway).

These studies will allow an understanding of the way in which GSH metabolism and related enzymes are factors involved in adriamycin resistance in this cell line.

Materials and methods

Cell culture

The MIP-101 human colorectal cell line was a gift from Dr P Thomas (Harvard Medical School, Boston, MA). The DLD-1 cell line was purchased from ATCC (CCL 221). These lines are poorly differentiated colon adenocarcinoma cell lines. The MIP-101 cells were 10 times as resistant to adriamycin as compared with DLD-1 cells.

Monolayer cultures of MIP-101 and DLD-1 were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2.

Subcultures of DLD-1 and MIP-101 were achieved by use of 0.25% trypsin plus 0.10% versene.

Drugs

Adriamycin was supplied by Farmitalia Carlo Erba (Milan, Italy). Biochemicals and their sources were: GSH and 1-chloro-2,4-dinitrobenzene (CDNB; Aldrich Chimie, Strasbourg, France); hydrogen peroxide (Merck, Darmstadt, Germany); γ-glutamyl-3-carboxy-4-nitroanilide (Boehringer Pharma, Mannheim, Germany); sodium acetate, disodium phosphate, orthophosphoric acid, Tris (Prolabo, Paris, France); methanol and acetonitrile (Carlo Erba). All other agents were from Sigma (St Louis, MO).

Chemosensitivity testing

Log-phase cultures were treated with adriamycin in the presence and absence of four chemosensitizers (BSO, verap, trifluo and tamox).

The cells were plated at 75×10^3 cells/ml in triplicate in a six-well plate. After incubation, drugs were added at the desired final concentration and the cells were incubated for 3 days. Adriamycin was added at increasing concentrations; cells were treated during 72 h with 200 μM BSO, 22.2 μM (10 μg/ml) verap, 6 μM trifluo or 6 μM tamox.

Cells in control and treated cultures were counted in a hemocytometer, and viability was determined by Trypan blue dye exclusion.

Detection of P-glycoprotein

The cell lines were pelleted by centrifugation, at 100 ***g*** during 10 min, then deposited as a monolayer cell smear on the slides. The cells were air dried, fixed in acetone and saturated by incubation at room temperature with PBSG pH 7 (phosphate buffer saline gelatine, 0.1% gelatine). After washing, the slides were then incubated at 37°C with the mouse monoclonal antibody C-219 (dilution: 1/25, 30 min, P-Glyco-CHECK C 219; Centocor Diagnostics, UK). This antibody recognizes an internal epitope. The cells were rinsed and incubated with biotinylated sheep anti-mouse second antibody (dilution 1/25, 30 min, Amersham, UK) or with biotinylated horse anti-mouse second antibody (dilution 1/25, 30 min, Centocor Diagnostics). Then, following five washes in PBSG, the streptavidin–fluorescein (dilution 1/50, Boehringer)–Blue Evans (dilution 1/10 000) complex was applied for 30 min at 37°C and five additional washes in PBSG were carried out. Slides were then

covered with antifading (DABCO) and immediately observed under a Zeiss axiophot microscope. Control slides were treated similarly except that the primary antibodies were omitted.

Enzyme assays

Total *intracellular GSH* (reduced and oxidized GSH) levels were measured using HPLC and electrochemical detection.[21]

GSH reductase was assayed at 30°C by the oxidized GSH-dependent oxidation of NADPH followed at 340 nm.[22]

GSH peroxidase (EC 1.11.1.9) activities were measured at 30°C by the appearance of the disulfide product (i.e. oxidized GSH) in a coupled assay procedure which required addition of exogenous GSH reductase (1 U/ml) and NADPH (0.1 mM), an adaptation of a published method.[23] *GSH transferases* were assayed on 1 mM GSH and a substrate common to the different transferases, CDNB (1 mM) at 340 nm. The type π GST isoenzyme was specifically assayed by using 0.2 mM ethacrinic acid and 0.25 mM GSH as substrates, and following the absorbance at 270 nm according to Habig and Jakoby.[24]

Established procedures have been used for the assay of other enzymes: *γ-glutamyl transpeptidase*[25] and *glucose-6-phosphate dehydrogenase*.[26]

Total protein determinations were carried out, following sonication of cells, by the bicinchoninic acid assay (BCA), using a kit from Pierce (Montluçon, France).[27]

Preparation of subcellular fractions from cell homogenates

Briefly, cells were harvested by trypsinization, pelleted by centrifugation at 100 ***g*** for 10 min, resuspended in 5 ml of PBS and centrifuged under the same conditions. The cell pellet was resuspended in 3 ml of PBS and homogenized by sonication at 4 C (three 7 s pulses with a 45 s interval between each pulse). Soluble proteins were obtained by submitting the cell homogenates to a 100 000 ***g*** centrifugation for 35 min and collecting the resulting supernatants.

Statistical analysis

All data are expressed as the mean ± SEM. The statistical significance between two means was determined by Student's *t*-test.

Results

Circumvention of adriamycin resistance in MIP-101 cells

The sensitivity of MIP-101 and DLD-1 cells to adriamycin was determined by counting in a hemacytometer and viability was evaluated by Trypan blue dye exclusion. The IC_{50} values corresponding to the concentration of adriamycin that reduced growth to 50% were 1 μM for MIP-101 and 0.1 μM for DLD-1, indicating an approximate 10-fold resistance.

The potentiating effects of verap, BSO, trifluo and tamox were evaluated. When verap was added at a final concentration of 10 μg/ml to MIP-101 and DLD-1 cells, the ratio of resistance obtained was 2. This result indicated that verap partially overcame adriamycin resistance in these colon carcinoma cell lines. Treatment with BSO (200 μM), trifluo (6 μM) and tamox (6 μM) also modulated the adriamycin sensitivity, but not to the same extent and did not confer full sensitivity to adriamycin. The ratio of resistance was 5, 5 and 4, respectively, as compared with 10 for untreated MIP-101.

Detection of P-glycoprotein

The detection of P-glycoprotein was realized by immunofluorescence using C219 as monoclonal antibody. MIP-101 cells, resistant *de novo* to adriamycin, showed an intense membrane immunoreaction to C219 and there was also some staining of the membrane in the parental (sensitive) DLD-1 cells suggesting P-glycoprotein expression in this cell line (Figure 1). The observation of P-glycoprotein in these two cell lines can be explained by the fact that DLD-1 and MIP-101 were colon carcinoma cell lines, and that the P-glycoprotein plays an important physiological role in these tissues. The apparent nuclear staining of the DLD-1 cells was non-specific and cannot be attributed to P-glycoprotein expression, the same staining being observed in the control cells (Figure 1). No immunoreaction can thus be observed in the control cells and no differences in the immunofluorescence staining were observed when the cells were treated by the different chemosensitizers (data not shown).

Levels of GSH and related enzymes in untreated cells

In an attempt to evaluate the effect of the four chemosensitizing agents on GSH content and

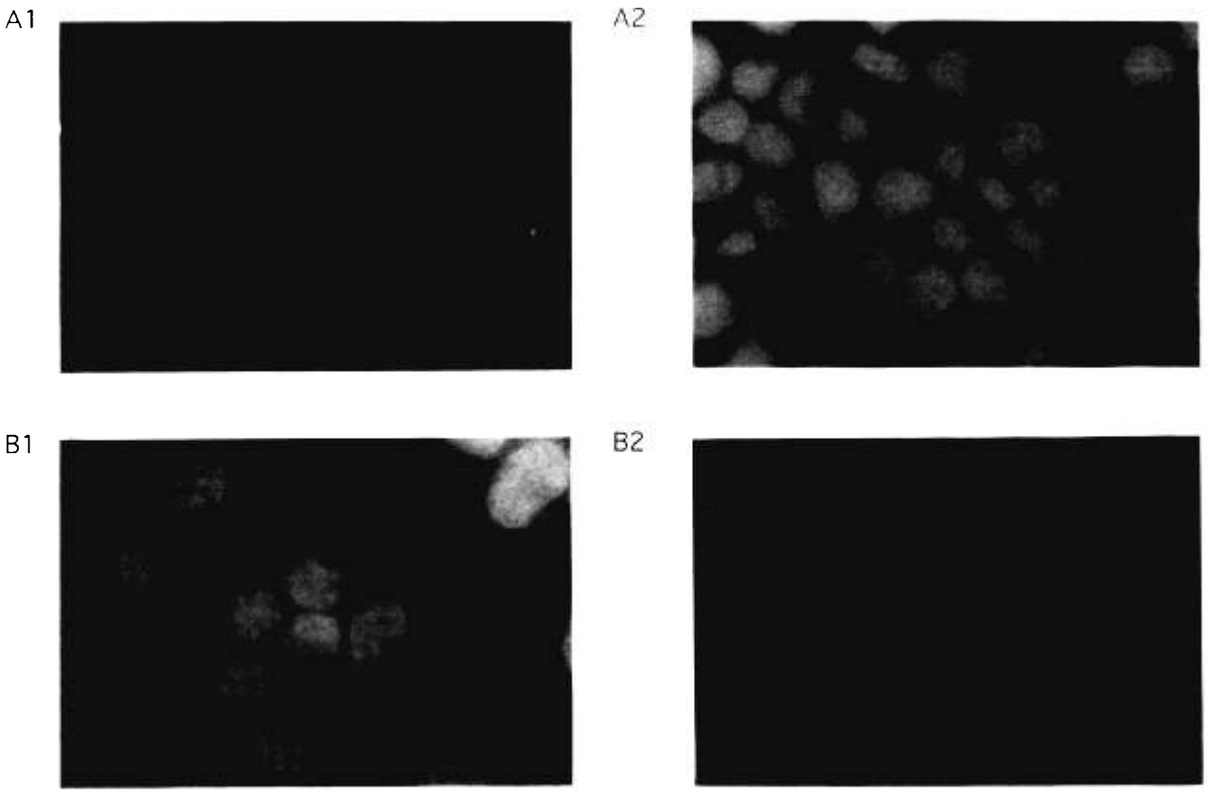

Figure 1. Indirect immunofluorescence staining of P-glycoprotein (by incubation with mouse monoclonal antibody C-219): in MIP-101 cells resistant *de novo* to adriamycin, test (A1) and control (A2); and in DLD-1 cells sensitive to adriamycin, test (B1) and control (B2).

GSH-related enzymes, we first studied these parameters in untreated MIP-101 and DLD-1 cells.

Analysis of the results of Table 1 showed that the adriamycin resistance in MIP-101 cells was significantly correlated ($p < 0.01$, Student test) with a 2-fold increase in the concentration of reduced GSH, in GSH reductase, with a 3- to 5-fold increase in GSH peroxidase activity and with a weak stimulation of glucose-6-phosphate dehydrogenase. On the other hand, a significant decrease ($p < 0.01$, Student test) of γ-glutamyl transpeptidase activity and GST activity using CDNB as substrate was observed.

Levels of GSH and related enzymes in treated cells

Effect of verap. Verapamil, a calcium channel antagonist, after 72 h incubation in the culture medium, induced an important decrease of reduced and oxidized GSH in both the sensitive and resistant cells (respective values of reduced GSH of 1195 ± 220 pmol/10^6 cells and 2464 ± 217 as compared with 3253 ± 314 and 8882 ± 881 for DLD-1 and MIP-101 (Table 2). The ratio MIP-101/DLD-1 decreased from approximately 20%.

Concerning the enzyme activities, this treatment resulted in a significantly increased activity ($p < 0.01$, Student test) of glucose-6-phosphate dehydrogenase (respective values of 151.45 ± 3.19 mU/mg protein and 409.11 ± 10.53 as compared with 114.77 ± 5.72 and 171.33 ± 7.03 for untreated DLD-1 and MIP-101 cells) coupled to a similar modification in GSH reductase activity (respective values of 59.96 ± 2.82 mU/mg protein and 89.47 ± 2.50 compared with 26.61 ± 0.98 and 53.55 ± 1.85 for untreated DLD-1 and MIP-101 cells). GSH peroxidase tested more specifically, when either *tert*-butyl hydroperoxide was used simultaneously with GSH as substrates of the enzyme activity, showed an increase activity in sensitive and resistant cells treated by verap. The most significant variation ($p < 0.01$, Student test) in the ratio MIP-101/DLD-1 cells was observed in

Table 1. Values of GSH (reduced and oxidized) and activities of GSH reductase, transferases, peroxidases, γ-glutamyl-transpeptidase, glucose-6-phosphate dehydrogenase in DLD-1 and MIP-101 cell lines. The last column shows the ratio of the activity between cells resistant (MIP-101) and sensitive (DLD-1) to adriamycin

	DLD-1	MIP-101	MIP/DLD
Glutathione			
GSH	3253 ± 314	8882 ± 881	2.73
GSSG	338 ± 112	450 ± 129	1.33
GSR	26.61 ± 0.98	53.55 ± 1.85	2.01
GST			
CDNB	145.81 ± 3.24	92.93 ± 1.00	0.64
ECA	12.73 ± 1.07	10.94 ± 1.01	0.86
GPX			
COOP	0.96 ± 0.10	5.11 ± 0.39	5.32
Tbut	1.69 ± 0.24	5.78 ± 0.77	3.42
γ-GT	59.28 ± 2.51	11.59 ± 0.56	0.19
G-6-PDH	114.77 ± 5.72	171.33 ± 7.03	1.49

For GSH content, the values represent the means ± SEM (pmol/10^6 cells) of eight determinations. For the different enzymes activities, the results are expressed as mU/mg protein and are means ± SEM of at least two to three separate experiments, each done in triplicate. Abbreviations: GSR, glutathione reductase; GST, glutathione transferase; CDNB, chlorodinitrobenzene; ECA, ethacrinic acid; GPX, glutathione peroxidase; COOP, cumene hydroperoxide; Tbut, *tert*-butyl hydroperoxide; γ-GT, γ-glutamyl-transpeptidase; G-6-PDH, glucose-6-phosphate dehydrogenase.

the GSH peroxidase activity assayed using cumene hydroperoxide as substrate, i.e. glutathione peroxidase activity and peroxidase activity of GST: a value of 1.77 was obtained compared with 5.32 for untreated cells. This result was explained mostly by increased activity of GSH peroxidase in the verap treated cells (1.91 ± 0.29 mU/mg protein as compared with 0.96 ± 0.10).

Except for glucose-6-phosphate dehydrogenase, the ratio MIP-101/DLD-1 of the different enzyme activities tended to decrease, thus contributing to the reversion of chemoresistance.

Effect of BSO. BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase, was incubated with colon carcinoma cell lines at a dose of 200 μM. This treatment resulted in nearly complete depletion of intracellular GSH, which reached 5% of the value of the untreated cells after 72 h (value of reduced GSH less than 100 pmol/10^6 cells).

The enzymes most strongly affected by this product were the GSH peroxidases, and the observed decrease in the MIP-101 cells was of the order of 30% (Table 3; respective values of 1.91 ± 0.29 mU/mg protein and 3.35 ± 0.36 compared with 0.96 ± 0.10 and 5.11 ± 0.39 for untreated DLD-1 and MIP-101 cells using cumene hydroperoxide and GSH as substrates, and respective values of 1.94 ± 0.29 mU/mg protein and 3.94 ± 0.36 as compared with 1.69 ± 0.24 and 5.78 ± 0.47 for untreated DLD-1 and MIP-101 cells using *tert*-butyl hydroperoxide and GSH as substrates). Concerning the GSH reductase and glucose-6-phosphate dehydrogenase activities, we observed a significantly increased activity ($p < 0.01$, Student test) in the MIP-101 treated cells and in the sensitive-treated cells; however, the increase was more important in the sensitive than in the resistant cells, inducing a decreased ratio of

Table 2. Effect of 10 μg/ml of verap on GSH and related enzymes in MIP-101 and DLD-1 cell lines

	DLD-1	MIP-101	DLD-1/verap	MIP-101/verap	MIP-verap/DLD-verap
Glutathione					
GSH	1	2.73	0.36	0.75	2.08
GSSG	1	1.33	1.29	0.89	0.69
GSR	1	2.01	2.25	3.35	1.49
GST					
CDNB	1	0.64	1.26	0.82	0.65
ECA	1	0.86	1.14	0.48	0.42
GPX					
COOP	1	5.32	2.98	5.27	1.77
Tbut	1	3.42	1.43	4.96	3.47
γ-GT	1	0.19	1.20	0.18	0.15
G-6-PDH	1	1.49	1.32	3.56	2.70

Results are expressed as a ratio between the activity of the cells treated (MIP-101 and DLD-1) or untreated (MIP-101) by comparison with the activity of the DLD-1 untreated cells to which a value of 1 is given. The last column shows the ratio of the activity between resistant (MIP-101) and sensitive (DLD-1) cells treated by verap. For GSH content, the values represent the mean of four determinations and for the enzyme activities the mean of at least two to three separate experiments, each done in triplicate. See Table 1 for abbreviations.

Table 3. Effect of 200 μM of BSO on GSH and related enzymes in MIP-101 and DLD-1 cell lines

	DLD-1	MIP-101	DLD-1/BSO	MIP-101/BSO	MIP-BSO/DLD-BSO
Glutathione					
GSH	1	2.73	—	—	—
GSSG	1	1.33	—	—	—
GSR	1	2.01	2.61	3.22	1.23
GST					
CDNB	1	0.64	1.54	0.64	0.42
ECA	1	0.86	1.21	0.72	0.60
GPX					
COOP	1	5.32	1.99	3.49	1.75
Tbut	1	3.42	1.15	2.33	2.03
γ-GT	1	0.19	1.17	0.15	0.13
G-6-PDH	1	1.49	1.72	1.89	1.10

Results are expressed as a ratio between the activity of the cells treated (MIP-101 and DLD-1) or untreated (MIP-101) by comparison with the activity of the DLD-1 untreated cells to which a value of 1 is given. The last column shows the ratio of the activity between resistant (MIP-101) and sensitive (DLD-1) cells treated by BSO. For GSH content, the values represent the mean of four determinations and for the enzyme activities the mean of at least two to three separate experiments, each done in triplicate. See Table 1 for abbreviations.

MIP-101/DLD-1 cells. Indeed, if we considered GSH reductase activity, respective values of 69.73 ± 1.99 mU/mg protein and 85.93 ± 4.31 as compared with $26.61 + 0.98$ and 53.55 ± 1.85 for untreated DLD-1 and MIP-101 cells were obtained. The DLD-1 treated cells also showed increased activities of GST and γ-glutamyl-transpeptidase and, conversely, the MIP-101-treated cells presented decreased activities of the same enzymes. All the ratios of MIP-101/DLD-1 cells thus decreased for the treated cells as compared with untreated cells and this phenomenon could contribute, as for verap, to an inhibition of the mechanisms of detoxification of adriamycin and an improved efficiency of this anti-cancer drug.

Effect of trifluo. Trifluo, a calmodulin antagonist, was incubated with the MIP-101 and DLD-1 cells at a concentration of 6 μM. This chemosensitizing agent induced a very important decrease of GSH (respective values of reduced GSH of 264 ± 101 pmol/10^6 cells and 1934 ± 373 compared with 3253 ± 314 and 8882 ± 881 for DLD-1 and MIP-101). This modification being more significant for the sensitive cells, the ratio MIP-101/DLD-1 was, by contrast to verap treatment, increased: 7.37 compared with 2.73 (untreated cells) and 2.08 (verap treated cells) (Table 4).

The most significant ($p < 0.01$, Student test) enzyme modification observed in the treated cells referred to the π isoenzyme GST in the MIP-101

Table 4. Effect of 6 μM of trifluo on GSH and related enzymes in MIP-101 and DLD-1 cell lines

	DLD-1	MIP-101	DLD-1/trifluo	MIP-101/trifluo	MIP-trifluo/DLD-trifluo
Glutathione					
GSH	1	2.73	0.08	0.59	7.37
GSSG	1	1.33	4.50	1.09	0.24
GSR	1	2.01	0.81	2.18	2.69
GST					
CDNB	1	0.64	1.21	0.65	0.54
ECA	1	0.86	0.66	0.39	0.59
GPX					
COOP	1	5.32	4.36	5.08	1.16
Tbut	1	3.42	2.03	2.50	1.23
γ-GT	1	0.19	0.82	0.16	0.19
G-6-PDH	1	1.49	1.32	1.99	1.51

Results are expressed as a ratio between the activity of the cells treated (MIP-101 and DLD-1) or untreated (MIP-101) by comparison with the activity of the DLD-1 untreated cells to which a value of 1 is given. The last column shows the ratio of the activity between resistant (MIP-101) and sensitive (DLD-1) cells treated by trifluo. For GSH content, the values represent the mean of four determinations and for the enzyme activities the mean of at least two to three separate experiments, each done in triplicate. See Table 1 for abbreviations.

Table 5. Effect of 6 μM of tamox on GSH and related enzymes in MIP-101 and DLD-1 cell lines

	DLD-1	MIP-101	DLD-1/tamox	MIP-101/tamox	MIP-tamox/DLD-tamox
Glutathione					
GSH	1	2.73	0.72	1.21	1.68
GSSG	1	1.33	2.76	1.69	0.61
GSR	1	2.01	1.30	0.74	0.57
GST					
CDNB	1	0.64	1.48	0.67	0.45
ECA	1	0.86	0.53	0.80	1.51
GPX					
COOP	1	5.32	2.97	5.02	1.69
Tbut	1	3.42	1.33	3.25	2.44
γ-GT	1	0.19	1.01	0.14	0.14
G-6-PDH	1	1.49	1.47	1.49	1.01

Results are expressed as a ratio between the activity of the cells treated (MIP-101 and DLD-1) or untreated (MIP-101) by comparison with the activity of the DLD-1 untreated cells to which a value of 1 is given. The last column shows the ratio of the activity between resistant (MIP-101) and sensitive (DLD-1) cells treated by tamox. For GSH content, the values represent the mean of four determinations and for the enzyme activities the mean of at least two to three separate experiments, each done in triplicate. See Table 1 for abbreviations.

cells with a 2-fold decrease (5.02 ± 0.35 mU/mg protein compared with 10.94 ± 1.01). A lower ratio of MIP-101/DLD-1 treated cells was shown for the GSH peroxidase activity (ratio of 1.16 and 1.23 using, respectively, cumene hydroperoxide and GSH as substrates or *tert*-butyl hydroperoxide and GSH compared with 5.32 and 3.42). These results were explained by the increased activity in the DLD-1 cells (respective values of 4.19 ± 0.28 mU/mg protein and 3.43 ± 0.28 compared with 0.96 ± 0.10 and 1.69 ± 0.24 for untreated cells using cumene hydroperoxide and GSH as substrates or *tert*-butyl hydroperoxide and GSH). These results are in agreement with the results obtained with verap and BSO which indicate a trend to the reversion of chemoresistance. Nevertheless, the ratio of MIP-101/DLD-1 for the GSH reductase, the glucose-6-phosphate dehydrogenase and the γ-glutamyl-transpeptidase activities was not affected by this treatment, as opposed to the results obtained for the verap or BSO treated cells.

Effect of tamox. Tamox, incubated at a concentration of 6 μM during 72 h, induced a decrease of reduced GSH which was more significant in the resistant cells (respective values of reduced GSH of 2363 ± 512 pmol 10^6 cells and 3951 ± 591 as compared with 3253 ± 314 and 8882 ± 881 for DLD-1 and MIP-101), thus resulting in a lower ratio of MIP-101 DLD-1, i.e. 1.68 compared with the ratio of 2.73 for the untreated cells (Table 5).

The GSH reductase activity in the MIP-101 cells was significantly diminished ($p < 0.01$, Student test) (value of 39.57 ± 2.34 mU/mg protein compared with 53.55 ± 1.87 for untreated cells); this led to a ratio of resistant/sensitive cells of only 0.57. The ratio of MIP-101/DLD-1 cells, for the other enzyme activities, was also decreased, except the GST π isoenzyme. These modifications for GSH peroxidases, GST (using CDNB as substrate) and glucose-6-phosphate dehydrogenase resulted from increased activities in the DLD-1 cells; indeed, we reported for GSH peroxidase values of 2.85 ± 0.26 and 2.25 ± 0.36 compared with 0.96 ± 0.10 and 1.69 ± 0.24 using, respectively, cumene hydroperoxide or *tert*-butyl hydroperoxide as substrate concomitantly with GSH. Tamox treatment also induced a reduced activity of γ-glutamyl-transpeptidase in the MIP-101 treated cells (value of 8.39 ± 0.36 mU/mg protein compared with 4.59 ± 0.50), the ratio being also decreased. All these variations tended to the reversal of chemoresistance to adriamycin.

Discussion

Several mechanisms for the development of MDR or pleiotropic resistance have been identified. Previous studies have defined overexpression of MDR gene (*mdr*1) mRNA and its protein product as well as overexpression of GSH transferases and or peroxidases, modification of topoisomerase II, altered sub-cellular adriamycin accumulation, and failure of drug uptake or activation as playing potentially important roles in the development of resistance to adriamycin. P-glycoprotein represents

an efflux transporter which extrudes a broad spectrum of anti-cancer drugs from the cell; however, it is unlikely to be the sole mechanism of resistance. As shown by Batist,[28] an important modification of GST activity can occur in human breast carcinoma (MCF-7) cell lines resistant to adriamycin. Other studies have also emphasized the role of GSH and related enzymes in chemoresistance to adriamycin.[12,29-31] This phenomenon is, however, not a general result and there are also conflicting results concerning these parameters.[32]

Drug resistance is thus a complex process involving many alterations in tumor biochemistry. This manuscript examines the effects of a number of chemosensitizers on cellular sensitivity to adriamycin of a colon carcinoma cell line (MIP-101) and its sensitive counterpart (DLD-1) by analysis of GSH and related enzymes.

The MIP-101 cells, *de novo* adriamycin-resistant cells, contain glycoprotein P-170 detected by immunofluorescence. MDR can be inhibited by a variety of agents. Their activity as MDR-reversing agents has, in general, nothing to do with their stated pharmacological effects. Presentation of adriamycin in liposomes induces a partial reversal of adriamycin resistance in MDR cell lines as well as the utilization of a variety of agents.[20,33-39]

In this study, we have treated MIP-101 and DLD-1 cells by a number of chemosensitizing agents (verap, BSO, trifluo and tamox). These compounds do not possess any significant structural features in common, are responsible of various pharmacological effects,[40,41] partially restore adriamycin sensitivity and induce no modification of P-glycoprotein whatever the treatment.

For each treatment with GSH, its content was substantially decreased. BSO, due to its inhibitory properties, induced a very important decrease in cellular glutathione content (greater than 95%); this modification has also been observed in cancer cells by Kramer *et al.*[12] and Batist *et al.*[42] Nevertheless, this compound was not the more effective chemosensitizer. If we consider trifluo, this treatment was more effective at decreasing the concentration of reduced GSH in DLD-1 cells (92% decrease) compared with MIP-101 cells (78% decrease), yet trifluo produced approximately the same relative degree of sensitization as tamox, which decreased GSH in DLD-1 and MIP-101 cells by only 27 and 56%, respectively. No relationship could thus be established between drug sensitivity and GSH content, verap being the more effective chemosensitizing agent and inducing decreases of 63 and 72% in DLD-1 and MIP-101 cells.

Among the different enzymes (GSH reductase, transferases, peroxidases, γ-glutamyl-transpeptidase and glucose-6-phosphate dehydrogenase) analyzed in this study, the γ-glutamyl-transpeptidase showed minor modifications whatever the treatment. This enzyme catalyzes the transfer of the γ-glutamyl moiety of GSH and other γ-glutamyl compounds to a variety of amino acids and peptides. The reaction catalyzed by this enzyme is of major importance in the γ-glutamyl cycle, a metabolic pathway that accounts for the enzymatic synthesis and degradation of GSH. In general, the activities were modified in the resistant and sensitive cells so that a decreased ratio of MIP-101/DLD-1 treated cells was observed; the different treatments induced some modifications in the enzymes activities but no general trend could be observed. Tamox was the compound which induced less modifications in the enzyme activities for the sensitive or treated cells (GST, GSH peroxidases and glucose-6-phosphatase dehydrogenase in MIP-101 cells and GSH reductase in DLD-1 cells were unchanged) but restored sensitivity to adriamycin more efficiently than trifluo and BSO (ratio of resistance of 4 as compared with 5). Compared with BSO, it also induced a decreased ratio of MIP-101/DLD-1 cells in nearly all the enzyme activities, these modifications being more important than for verap and trifluo.

In fact, GSH accounts for the majority of the intracellular non-protein sulfhydryl content and participates in many cellular functions, including protection from free radical damage, detoxification of xenobiotics and prevention of damage to proteins or DNA.[43] Indeed, GSH can react directly with anti-cancer drugs and is also a substrate for GSH reductase and transferase; GSH peroxidase can detoxify adriamycin by reacting with the hydroxyl radicals. Glucose-6-phosphate dehydrogenase interacts with these enzymes because it supplies reducing equivalents (NADPH) for the reduction of oxidized GSH, and its activation is an index of GSH redox cycling by GSH peroxidase. The interaction of GSH with adriamycin is probably related to the quinone/semi-quinone interconversion and this may or may not need enzymatic catalysis. It is claimed that hydroxyl radicals of adriamycin provide the substrate for GSH.[19,44-46]

The chemosensitizing agents, which are of very different structure, induce similar effects on GSH and related enzymes. All modifications of these parameters are factors contributing to the decrease of resistance. As these chemosensitizers did not confer full sensitivity, we cannot assert that GSH and related enzymes are the only factors contribu-

ting to chemoresistance; however, this study confirms the role of GSH and related enzymes in chemoresistance to adriamycin in addition to other factors such as glycoprotein P-170 present in these cells.

Conclusion

The concept of overcoming drug resistance is based on there being a synergistic interaction between the modulator and the cytotoxic agent. This manuscript reports on the effects of a number of unrelated chemosensitizers on cellular sensitivity of colon carcinoma cell lines to adriamycin. Treatments which sensitized the MDR cells decreased GSH levels and the activities of several GSH associated enzymes (e.g. GSH peroxidase, GSH reductase, etc.). These data support the conclusion that alterations in GSH and associated enzymes occur following treatment with various chemosensitizers. Nevertheless, it is optimistic at best to conclude definitively that these changes are the cause of the reversal of resistance.

It is also necessary to keep in mind that the use of modulators of P-glycoprotein cannot be critically assessed without taking into account the effects which these agents, if introduced at truly biologically effective concentrations, may have on normal physiological processes in which P-glycoprotein may play a part.

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