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Different mechanisms for γ-glutamyltransferase-dependent resistance to carboplatin and cisplatin

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Abstract

In this work, we investigated the effect of γ-glutamyltransferase (GGT) overexpression on cell viability after carboplatin treatment and compared with cisplatin. Carboplatin challenge of HeLa cells induced GGT and glutamate-cystine ligase (GCL) activities by 2- and 1.4-fold, respectively and concomitantly increased the intracellular reduced glutathione (GSH) level (1.5-fold). To study the role of GGT, HeLa-GGT cells, a stably transfected cell line overexpressing GGT (120–150 mU/mg protein) and the parental HeLa cells (10–15 mU/mg protein) were used. Both cell lines exhibited comparable viability ($IC_{50} \sim 150$ μM) after carboplatin treatment when cultured in standard (250 μM cystine) medium. Culture in low (50 μM) cystine medium resulted in a dramatic decrease (∼90%) of the intracellular GSH level and to a 2.5-fold increase of carboplatin cytotoxicity ($IC_{50} \sim 60$ μM). When GSH (50 μM) was included in the culture medium, only HeLa-GGT cells exhibited increased resistance to carboplatin. Using partially purified GGT from HeLa-GGT cells, we show that cisplatin forms adducts with cysteinylglycine, depending only on GGT activity whereas carboplatin did not efficiently react with cysteinylglycine. Thus, in this model system, GGT activity can affect platinum drugs cytotoxocity by two different ways: cisplatin can be detoxified extracellularly after reaction with the –SH group of cysteinylglycine; in the case of carboplatin, the supply of GSH precursors, initiated by GGT, increases the intracellular level of the tripeptide and provides enhanced defensive mechanisms to the cell.

Keywords: Cisplatin; Carboplatin; γ-Glutamyltransferase; GSH; Adducts; Resistance

1. Introduction

Glutathione (γ-glutamylcysteinylglycine, GSH) is a tripeptide that plays a central role in the cellular defense against oxidative stress and xenobiotics. These properties are linked to the ability of the thiol group of the cystine to react with electrophilic compounds and thus protect the cell from toxic electrophiles [1]. Intracellular level of GSH is often related to the response of cells to chemotherapeutics and increased GSH levels are often correlated with resistance to platinum-based drugs [2]. Cisplatin (*cis*-diamminedichloroplatinum (II), CDDP) can form an adduct with GSH by nucleophilic attack of the GSH thiolate anion [3], and this conjugate can be exported out of the cell by an ATP-dependent pump (GS-X) [3]. This mechanism reduces the cytotoxic potential of cisplatin. GSH has also a protective role against injuries by maintaining the dNTP pool needed for DNA repair and also by maintaining functional repair enzymes such as polymerase α [4]. The intracellular level and turnover of GSH depends on GCL activity, which catalyzes the initial and limiting step of its synthesis, and on the cystine supply which is depending on the availability of extracellular cystine and GSH.

GGT is a glycosylated membrane enzyme that initiates the cleavage of extracellular GSH, and thus involved in the supply of the precursors for its intracellular synthesis [5]. Therefore, cells with high GGT activity have an advantage for their cystine supply [6]. GGT level could be a limiting factor in GSH biosynthesis in accordance to Vincent *et al.* who have shown that the supply of cystine is more important

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Abbreviations: GSH, glutathione; GGT, γ-glutamyltransferase; GCL, glutamate-cystine ligase; GSTs, glutathione S-transferases; CDDP, *cis*-diamminedichloroplatinum (II); CBDCA, carboplatin; cysgly, cysteinylglycine; LC-DMEM, low cystine DMEM; HC-DMEM, high cystine DMEM; CDNB, 1-chloro-2,4-dinitrobenzene; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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than the GCL activity [7]. GGT activity was shown to be induced in cancer cell lines [8–10], and tumors overexpressing this enzyme have an advantage for their proliferation [11] and metastasis, *in vivo* [12]. Furthermore, GGT is induced in cells selected for their resistance to cisplatin [2,13]. By these properties, GGT could play an important role in the cellular response to platinum drugs, but the mechanisms involved are not yet elucidated.

In a previous study [14], we demonstrated that an increased GGT activity enhances the cell resistance to cisplatin only in the presence of extracellular GSH. The enhanced resistance was attributed to the extracellular conjugation of cisplatin to cysgly the latter being the product of GSH metabolism by GGT. In addition, the cisplatin resistance was not correlated with the intracellular GSH level.

The emergence of resistance to cisplatin contributed to the development of new drugs derivated from platinum such as carboplatin. Carboplatin (*cis*-diammine (1,1-cyclobutanedicarboxylato)-platinum (II), CBDCA) is an antineoplastic drug presenting an activity profile similar to cisplatin but a reduced toxicity and especially a reduced nephrotoxicity, which is the limiting factor to cisplatin therapy. Carboplatin is also submitted to cellular responses leading to a resistance. For example, carboplatin presents some cross-resistance with cisplatin in different cell lines and GSH can be associated with these phenomena [15].

In this work, we asked whether an overexpression of GGT could also influence the cellular toxicity of carboplatin. Using the cellular models HeLa and HeLa-GGT developed earlier [14], we provide evidence that GGT enhances the resistance to carboplatin by a different mechanism than to cisplatin, involving the intracellular GSH level.

2. Material and methods

2.1. Cell lines, culture conditions and treatments

The HeLa-GGT cell line was established after stable transfection by the vector pTRE-GGT of HeLa-Tet-off (Clontech). This vector contains the full length cDNA of human GGT [14].

Cells were grown in DMEM (Sigma, this medium contains 250 μM cystine and methionine, and will be referred as HC-DMEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (Boehringer) and 1% (v/v) antibiotic-antimycotic solution (Sigma). The DMEM without cystine and methionine (Sigma), was supplemented with 50 μM cystine and 50 μM methionine (Gibco) (this medium will be referred as LC-DMEM) and used for experiments in medium with low levels of sulfur-containing amino acids. Cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% CO_2.

5 mM carboplatin (Sigma) stock solution was prepared in H_2O and stored at −20° for 6 months.

5 mM cisplatin (Sigma) stock solution was prepared in 150 mM NaCl and stored at −80° for 6 months.

50 mM GSH (Sigma) stock solution was prepared in H_2O and stored at −80° for 6 months.

2.2. Measurement of GGT, GCL and GSTs activities

2.2.1. GGT activity

GGT activity was determined using L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine glygly as donor and acceptor substrates, respectively as previously described [16], and was expressed as mU/mg protein (nmol of 5-nitrobenzoate/min/mg protein).

GGT activity was also measured using GSH as donor substrate and glygly as acceptor. 150 μL of partially purified enzyme (Section 2.3) were incubated at 37° in a mixture containing 1 mM GSH, 5 mM glygly and 150 mM NaCl in 10 mM phosphate buffer pH 7.4. Samples were withdrawn at various times and the GSH and cysgly were quantified by HPLC (see methods described in Section 2.6). GGT activity was calculated from the linear part of the curve (nmol cysgly vs. time).

2.2.2. GCL activity

GCL activity was measured as described by Parmentier *et al*. [17] and expressed as nmol glucys/min/mg protein.

2.2.3. GSTs activity

GSTs activity of the two cell lines were determined using CDNB (Sigma) as substrate according to the method developed by Habig *et al*. [18].

Cells were cultured in 12-well plates in the appropriate medium before experiment. They were washed twice with cold PBS and scrapped in 400 μL of PBS. The suspension was sonicated and centrifuged at 12,000 *g* for 5 min. 100 μL of the supernatant were withdrawn to measure the total protein concentration. The remaining supernatant was supplemented with 1/10 (v/v) BSA (50 mg/mL) and GSTs activity of each sample was measured within 1 hr. 50 μL of 20 mM CDNB (Sigma), 400 μL of 0.2 M phosphate buffer pH 6.5, 50 μL of 20 mM GSH and 50 μL of the sample to analyze were mixed and the increase of the absorbance at 340 nm was continuously monitored for 5 min.

The activity, expressed in nmol CDNB/min/mg protein, was calculated using the molar absorption coefficient of the glutathione conjugate of CDNB at 340 nm (9600 M^{-1} cm^{-1}).

Protein concentration was determined using Biorad reagent (Coomassie Blue) according to the manufacturer's recommendations and using bovine serum albumin as standard.

2.3. Purification of GGT from HeLa-GGT cells

Recombinant human GGT was partially purified from HeLa-GGT cells by the following protocol. HeLa-GGT

cells, cultured under standard conditions, were scrapped and washed in PBS buffer. They were resuspended in PBS and papain was added to a final concentration of 0.05 mg papain per mg of cellular protein. The cell suspension was stirred for 2 hr at room temperature and overnight at +4°. Insoluble material was eliminated by centrifugation (20,000 *g*, 30 min, +4°) and the supernatant was applied to a DEAE-Sepharose gel, previously equilibrated with 50 mM Tris–HCl pH 8.0 buffer. After washing the unbound proteins with the same buffer, the enzyme was eluted by applying a linear NaCl gradient (0–0.5 M) in the equilibration buffer. Fractions exhibiting the highest enzymatic activity were pooled and concentrated and desalted using Centriprep 3 (cut off 3000 Da, Amicon) concentrators. The final preparation exhibited a GGT specific activity towards the synthetic substrate of 820 nmol/min/mg protein. The enzyme was aliquoted and stored at −80°.

2.4. Measurement of intracellular and extracellular thiols

2.4.1. Intracellular GSH

The cells were seeded in 6-well plates and cultured either in HC- or in LC-DMEM during 24 hr. Cells were then incubated for additional 48 hr in HC- or in LC-DMEM, in the absence or in the presence of 50 μM GSH, and were washed with cold PBS, scrapped in 500 μL of 3.3% (w/v) perchloric acid, and centrifuged at 12,000 *g* for 5 min at +4°. The supernatant was used for thiol measurement and the pellet for protein determination. Samples were kept at −80° until HPLC analysis [17].

2.4.2. Extracellular concentrations of GSH, cysgly and cystine

HeLa and HeLa-GGT cells were seeded in 24-well plates as previously described, they were incubated in LC-DMEM supplemented with 50 μM GSH. 200 μL aliquots of medium were sampled after 24 and 48 hr of incubation and mixed with 200 μL 3.3% (w/v) perchloric acid solution. Prior to HPLC analysis, samples were diluted 10-fold in 9‰ (w/v) NaCl containing 4 mM EDTA.

Quantification of extracellular thiols concentrations, GSH, cysgly and cystine, were performed using a reverse phase HPLC method including a reduction step of disulfide bonds with tri-*n*-butylphosphine and precolumn derivatization with a thiol-selective fluorogenic reagent 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide as previously described by Salazar *et al*. [19].

2.5. Survival assays

Viability was assessed by the MTT assay. HeLa or HeLa-GGT cells were plated in 24-well plates and cultured for 24 hr in the appropriate medium. Then, cells were incubated in 200 μL of medium containing the indicated concentrations of carboplatin. After additional 48 hr of culture, 20 μL MTT (5 mg/mL) were added to the cells, followed by incubation for 3 hr. The resulting formazan product was solubilized in 200 μL DMSO and was measured spectrophotometrically at 550 nm.

2.6. Analysis of adduct formation between platinum compounds and thiols

Platinum compounds at 0.5 mM were incubated for 90 min at 37° in the presence of 1 mM GSH, 5 mM glygly, 150 mM NaCl and purified GGT (activity = 330 nmol cysgly/min/mL) in 10 mM phosphate buffer pH 7.4.

Inhibition of GGT activity was performed by preincubating the purified enzyme with 50 μM acivicin for 30 min at 37°.

The HPLC system used to monitor the production of adducts included an Aquasil® C18 (3 μm) 150 mm × 3 mm i.d. column (Thermo Hypersil) eluted with a 10 mM phosphate buffer pH 2.4 containing 5 mM pentanesulfonic acid, at a flow rate of 0.5 mL/min. UV detection was operated at 215 nm. Retention times of cisplatin, glygly, acivicin, carboplatin, cysgly, GSH were 1.9, 2.8, 3.8, 4.5, 5.8 and 7.9 min, respectively. Retention times of oxidized forms of cysgly and GSH were obtained from isolated injections of stock solutions of cysgly and GSH oxidized by H_2O_2 (30% (w/w)) in a proportion of 1% (v/v) in the final sample and were 17.5 and 24.5 min, respectively.

Retention time of cysgly–cisplatin adduct was determined after injection of a solution of cisplatin (0.5 mM) and cysgly (1 mM) incubated at 37° for different periods. A peak was observed at a retention time of 7.1 min, increasing with the incubation time.

The peak observed at a retention time of 3.4 min was attributed to the compound γ-gluglygly because of its increase when GSH (1 mM) and glygly (5 mM) were incubated with purified GGT. Also, its hydrophilic status gives a high probability of a retention time approximately similar to glygly.

2.7. Statistical analysis

All assays were performed at least in triplicate, and the results were expressed as the mean and standard deviation (mean ± SD). Student's *t* paired test was used for statistical analysis.

3. Results

3.1. Effect of carboplatin treatment on GGT and GCL expression and on intracellular GSH

We have previously shown that a single and short exposure of HeLa cells to cisplatin results in an induction of GGT activity and in an increase in the intracellular GSH level [14]. In order to test whether an equivalent treatment

Table 1
Effect of carboplatin (CBDCA) treatment on GGT, GCL and GSH intracellular content

	Control cells	CBDCA treatment (50 μM, 48 hr)
GGT activity[a]	14.6 ± 0.5	26.6 ± 1.2*
GCL activity[b]	230 ± 29	319 ± 36*
Intracellular GSH (nmol/mg protein)	31.2 ± 2.7	42.1 ± 3.9*

Results are the mean ± SD of three independent experiments. (*) $P < 0.01$ vs. control cells; (a) nmol 5-nitrobenzoate/min/mg protein; (b) nmol γ-glucys/mg protein/min.

with carboplatin has similar effects, HeLa cells were challenged with 50 μM of the drug for 48 hr and GGT, GCL, and GSH levels were measured. GGT and GCL activities were induced by carboplatin (2- and 1.4-fold, respectively) (Table 1). The induction of these GSH-related enzymes is well correlated with the increase of intracellular GSH level (1.5-fold).

Thus, as in the case of cisplatin, an early response of HeLa cells to carboplatin is the induction of GSH related enzymes, which could play an important role in the detoxification of this drug.

3.2. Effect of GGT expression on intracellular GSH levels

In order to study the effect of GGT activity on the cytotoxicity of carboplatin, we used the HeLa-GGT cell line [14]. HeLa-GGT cells express 150 mU GGT/mg protein, a level approximately 10-fold that of their untransfected counterparts (14 mU/mg protein). Both cell lines were cultured in the usual culture medium (HC-DMEM, 250 μM cystine), or in a thiol-poor medium (LC-DMEM, 50 μM cystine) with or without 50 μM GSH.

Growth of both cell lines in HC- and in LC-DMEM is shown in Fig. 1. HeLa and HeLa-GGT cells were seeded in 24-well plates either in HC- or in LC-DMEM and the number of viable cells was estimated after 24 and 48 hr of culture using the MTT assay. We found no significant difference in the cell populations after 24 hr of culture either in HC- or in LC-DMEM. The cell population of both cell lines increased almost 3-fold from 24 to 48 hr of culture in the HC-DMEM medium. When LC-DMEM medium was used for the HeLa-GGT cells, the cell population increased 2.6-fold during the same time period while for the HeLa cells, it doubled. Thus, although a small decrease in the cell growth was observed under these conditions, both cell lines were able to proliferate, despite their low intracellular GSH level (see below).

Table 2
HeLa and HeLa-GGT intracellular GSH levels in different culture conditions

Culture medium	Time of culture (hr)	GSH (nmol/mg protein)	
		HeLa	HeLa-GGT
HC-DMEM	24	39.2 ± 7.7	36.2 ± 0.7
	48	31.0 ± 2.7	27.0 ± 2.6
LC-DMEM	24	2.2 ± 0.6	2.7 ± 0.5
	48	1.1 ± 0.3	0.4 ± 0.1
LC-DMEM + GSH 50 μM	24	6.1 ± 1.2** [a]	19.1 ± 3.0** [a,b]
LC-DMEM + GSH 50 μM	48	12.9 ± 1.0** [a]	9.5 ± 1.2** [a,b]

Results are the mean ± SD of three independent experiments. (**) $P < 0.005$: (a) vs. the same cell line cultured in LC-DMEM the same time; (b) vs. HeLa cells cultured in the same medium the same time.

Intracellular level of GSH in both cell lines was measured after 24 or 48 hr of culture in the above media and the results are summarized in Table 2. As expected, and in accordance to our previously published results, GGT overexpression did not influence the intracellular GSH content after 24 hr of culture at a high concentration of cystine (39.2 ± 7.7 nmol/mg protein and 36.2 ± 0.7 nmol/mg protein in HeLa and HeLa-GGT, respectively). After 48 hr of culture, the intracellular GSH level of both cell lines decreased. However, both cell lines exhibited similar

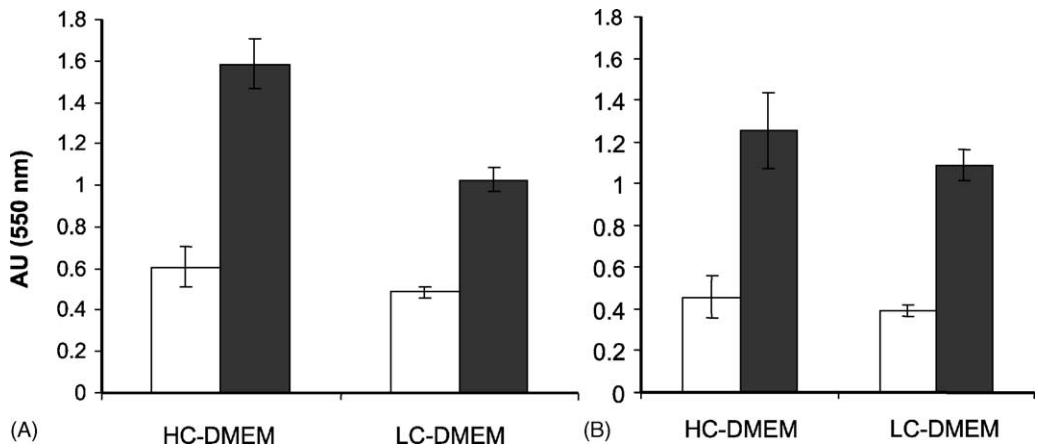

Fig. 1. Comparative growth of HeLa and HeLa-GGT in HC- and in LC-DMEM. Cells were seeded in 24-well plates and were incubated for 48 hr in HC-DMEM (250 μM cys) and in LC-DMEM (50 μM cys). Viability of HeLa (A) and HeLa-GGT cells (B) in both media was evaluated at $t = 24$ hr (□) and at $t = 48$ hr (■) of growth, by the MTT assay. Results are the mean ± SD of four independent experiments.

contents of GSH (31.0 ± 2.7 nmol/mg protein and 27.0 ± 2.6 nmol/mg protein for HeLa and HeLa-GGT, respectively).

Culture in LC-DMEM (low cystine levels) for 24 hr resulted in a dramatic decrease (∼90%) of the intracellular GSH in both cell lines. After 48 hr of culture in the same medium, the intracellular level of GSH decreased further to barely detectable levels (1.1 ± 0.3 nmol/mg protein and 0.4 ± 0.1 nmol/mg protein for HeLa and HeLa-GGT, respectively). When extracellular GSH was added to the LC-DMEM, cells overexpressing GGT were able to restore intracellular GSH (19.1 ± 3.0 nmol/mg protein) to 50% of the normal level, whereas HeLa cells restore it to 15% (6.1 ± 1.2 nmol/mg protein) of the normal level. After 48 hr in LC-DMEM medium, the intracellular GSH was decreased in HeLa-GGT cells (9.5 ± 1.2 nmol/mg protein), but it continued to increase in HeLa cells (12.9 ± 1.0 nmol/mg protein).

We then examined the thiol composition of the extracellular medium when the cell lines were cultured in LC-DMEM supplemented with 50 μM GSH. Culture medium samples were withdrawn after 24 and 48 hr of culture and GSH, cys and cysgly concentrations were determined by HPLC. As shown in Fig. 2A, after 24 hr of culture, GSH concentration in the HeLa-GGT medium was under the detection limit of the method while cysgly and cys concentrations were 9.9 ± 1.5 μM and 23.2 ± 3.7 μM, respectively. In contrast, HeLa cells were able to consume only a small amount (∼15 μM) of GSH in the same time period. After 48 hr of culture, the concentration of GSH in the extracellular medium of HeLa cells was about 20 μM and it was still undetectable in the case of HeLa-GGT cells. A small amount of cysgly (5–7 μM) and a significant amount of cystine (∼20 μM) were present in the same time period. These results indicate that under these conditions cys availability cannot be considered as a limiting factor for cell growth, as still considerable amounts are present after 48 hr of culture. These results also support the fact that overexpression of GGT allows cells to use the extracellular GSH, as a source of cys for the intracellular synthesis of the tripeptide, faster than cells with a low GGT activity. Extracellular GSH was rapidly used by HeLa-GGT cells, and after 24 hr, no more GSH was available. This catabolism led to an increase of the intracellular GSH level after 24 hr of culture and to a decrease after 48 hr (Table 2). On the contrary, cells expressing the enzyme at a lower level use extracellular GSH slowly during the 48 hr of culture. Thus, in HeLa cells, intracellular GSH concentration slowly but steadily increased during the same culture periods (Table 2).

3.3. Cytotoxicity of carboplatin

We then studied, under the same culture conditions, the cytotoxic effect of carboplatin on the two cell lines after exposed to different concentrations of the drug for 48 hr (the cytotoxicity after 24 hr treatment was negligible, data not shown). Viability was evaluated using the MTT assay.

As shown in Fig. 3, carboplatin was equally toxic to HeLa and HeLa-GGT cells in the HC-DMEM medium (IC_{50} ∼ 150 μM). When treatments were performed in LC-DMEM in which the intracellular levels of GSH are lower in both cell lines (Table 2), the IC_{50} was decreased to 60 μM, suggesting that the cells were more sensitive to the antiproliferative action of the drug. Thus, the intracellular level of GSH seemed to be crucial for the cytotoxicity of carboplatin.

In order to test whether GGT activity and the presence of extracellular GSH affects cytotoxicity, HeLa and HeLa-GGT cells were exposed to 75 μM carboplatin in LC-DMEM without GSH and with 5 or 50 μM GSH (Fig. 4).

The viability of cells grown in HC-DMEM in the presence of 75 μM carboplatin was 70% that of untreated cells, and was designated as ‘‘control’’, or 100%. In the presence of exogenous GSH (5 and 50 μM), HeLa-GGT cells were more resistant to 75 μM carboplatin (2- and

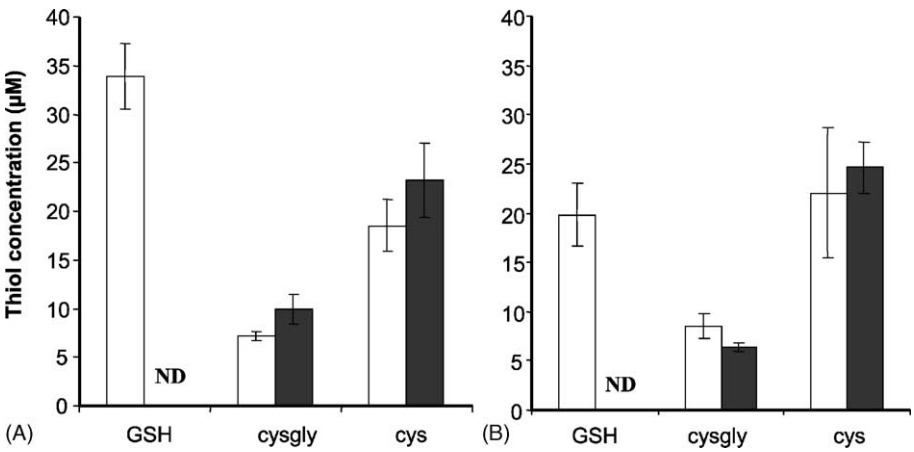

Fig. 2. Thiols composition of the extracellular medium of HeLa and HeLa-GGT cells. Cells were seeded in 24-well plates and incubated with LC-DMEM containing 50 μM GSH. After 24 hr (A) and 48 hr (B) of culture, 200 μL of extracellular medium of HeLa (□) and HeLa-GGT (■) cells were withdrawn and analyzed by HPLC according to Salazar *et al.* [19]. Results are the mean ± SD of three independent experiments.

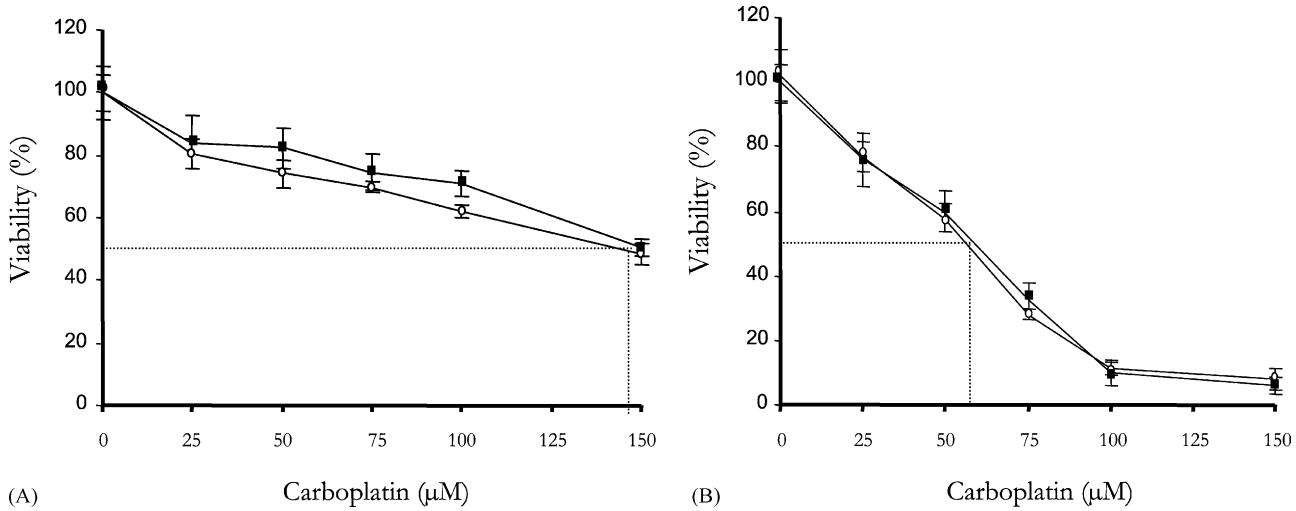

Fig. 3. Carboplatin cytotoxicity on HeLa and HeLa-GGT cells. HeLa (○) and HeLa-GGT (■) cells were seeded in 24-well plates and were incubated for 48 hr in the presence of indicated concentrations of carboplatin. Viability was measured by the MTT assay: (A) cells were cultured in HC-DMEM (250 μM cys); (B) cells were cultured in LC-DMEM (50 μM cys). Results expressed as percentage of untreated control cells are the mean ± SD of four independent experiments.

2.8-fold, respectively) than those grown without GSH, whereas the resistance of HeLa cells did not increase. These results can be interpreted as follows: carboplatin is hydrolyzed slowly within the cell to its active form, explaining the delay in the onset of cytotoxicity (48 hr) as compared to that of cisplatin (24 hr) [14]. Although we did not determine the time point at which the active form of carboplatin reached a level sufficient to induce cellular death, we suggest that because a correlation existed between the intracellular level of GSH and the cytotoxic effect of carboplatin, it was the high level of GSH in HeLa-GGT cells at 24 hr that was responsible for the increased resistance to carboplatin.

Thus, the mechanisms of GGT-dependent resistance to cisplatin and to carboplatin seem to differ: detoxification of cisplatin involves extracellular degradation of GSH by GGT, production of cysgly, which in turn reacts with cisplatin to form the non-toxic adduct cysgly–cisplatin in the medium, whereas the detoxification of carboplatin, involves intracellular GSH.

3.4. GGT-dependent formation of adducts

The HPLC system previously described [14] was modified in order to improve the separation between the adducts, the platinum drugs and between GSH and cysgly. As shown in Fig. 5A (zero time), the compounds were well separated.

In order to test whether GGT can catalyze the formation of cysgly–cisplatin and cysgly–carboplatin adducts, either

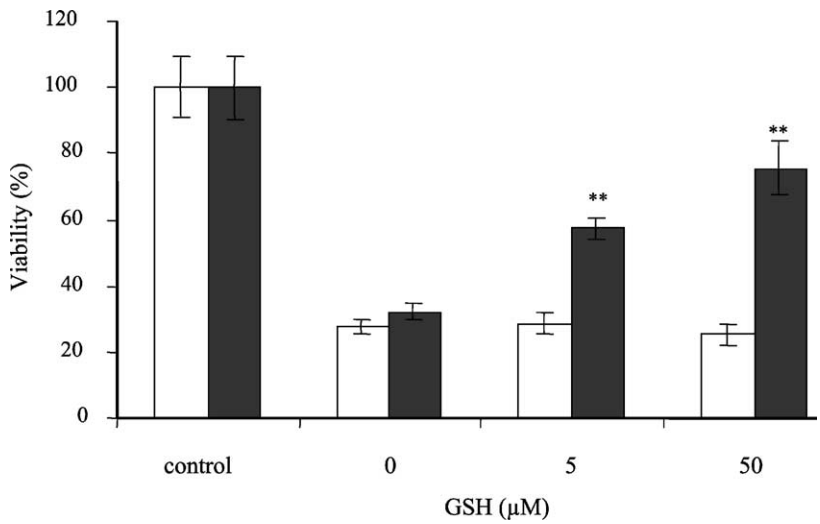

Fig. 4. Effect of extracellular GSH concentration level on carboplatin cytotoxicity of HeLa and HeLa-GGT cells. HeLa (□) and HeLa-GGT (■) cells were cultured in LC-DMEM supplied with the indicated GSH concentrations and were treated with 75 μM carboplatin during 48 hr. The percent viability corresponding to the mean ± SD of four independent experiments is compared to the control cells (100%) which are cultured in the HC-DMEM and treated as previously. (**) $P < 0.005$ vs. control cells.

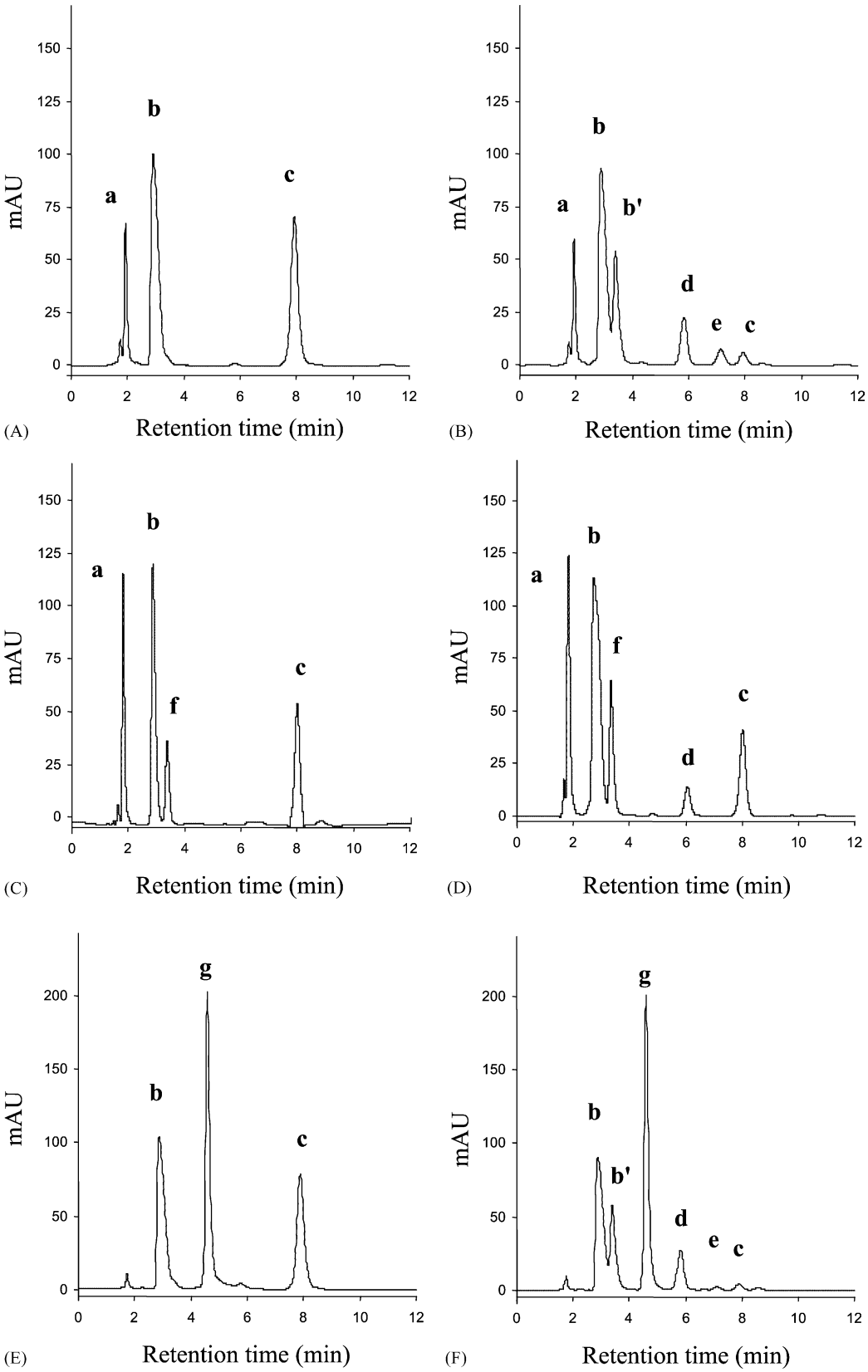

Fig. 5. Formation of thiol–platinum adducts in the presence of GSH and GGT. 0.5 mM of platinum compounds (CDDP and CBDCA) were incubated at 37° with 1 mM GSH, 5 mM glygly in the presence of purified GGT (330 nmol cysgly/mL/min). Formation of cysgly–cisplatin adduct was evaluated at time: 0 min (A) and 90 min (B) and with acivicin as GGT inhibitor (C and D). Formation of carboplatin–cysgly adduct was also evaluated at time: 0 min (E) and 90 min (F). Retention times are 1.9, 2.8, 3.4, 3.8, 4.5, 5.8, 7.1 and 7.9 min, respectively, for cisplatin (a), glygly (b), γ-gluglygly (b′), acivicin (f), carboplatin (g), cysgly (d), cysgly–cisplatin adduct (e) and GSH (c).

drug was incubated in a reaction mixture containing GSH, GGT and glygly. The enzyme, partially purified from HeLa-GGT cells, was confirmed to be GGT by the almost complete inhibition of the activity (330 nmol/mL/min) with the irreversible and specific GGT inhibitor acivicin at 50 μM (data not shown).

After a 90 min incubation at 37° (Fig. 5B), more than 90% of GSH were consumed, and two new peaks corresponding to cysgly (peak d) and cysgly–cisplatin adduct (peak e) appeared. Pretreatment with 50 μM acivicin (peak f) abolished the formation of cysgly and of the adduct (Fig. 5C and D), demonstrating that the formation of the adduct was dependent on the catalytic activity of GGT.

Similar incubation with carboplatin (peak g) (Fig. 5F), did not result in the appearance of a peak corresponding to an adduct, strongly suggesting that carboplatin does not react with cysgly, and indicating that in contrast to cisplatin, an extracellular mechanism involving GSH and GGT is not responsible for the increased resistance of HeLa-GGT cells. However, a small peak (close to the detection limit) with a retention time similar to the cysgly–cisplatin adduct was present. This adduct could arise from the reaction of the hydrolyzed carboplatin (identical to hydrolyzed cisplatin) which can react with cysgly.

4. Discussion

In the cisplatin structure, a central platinum atom is surrounded by two chlorine atoms and two ammonia molecules. The active form of cisplatin is obtained by hydrolysis and loss of the chlorine atoms to produce a highly electrophilic positive ion, which is able to interact with DNA among other cellular components. In carboplatin, the 1,1-cyclobutanedicarboxylate moiety replaces the two chlorine atoms [20], and the location of the platinum atom in a six-membered ring, results in a slow hydrolysis rate to cisplatin and activation. This slower rate leads to the differences in pharmacokinetics and cytotoxicity between the two drugs. In human, carboplatin has a wide antitumoral activity with proven efficiency in ovarian cancer, germ cells tumors, breast cancer, soft-tissue sarcoma, head and neck cancer, urinary tract tumors and brain tumors [21]. One of the main advantages of carboplatin as compared to cisplatin is its less important nephrotoxicity [22].

However, the emergence of acquired resistance to carboplatin is the main drawback in chemotherapy. The resistance to carboplatin could be multifactorial, and could involve the same mechanisms as those leading to the resistance to cisplatin. Cross resistance between these drugs, e.g. increase of the efflux of platinum from cells [23], or a decrease of the energy-dependent uptake [24] have been described. In addition, overexpression of the anti-apoptotic proteins Bcl-2 or BclXL was found to be responsible for the resistance to cisplatin [25] in ovarian cancer cell line, and to carboplatin in squamous cell carcinoma [26]. The *p53* tumor suppressor gene plays a central role in cell cycle regulation and induction of apoptosis. Mutations of *p53* have been implied in the resistance to cisplatin [27] or carboplatin [28], probably by inhibition of the apoptotic pathway.

However, one of the most important factors in the resistance of cells to cisplatin and carboplatin is the role of intracellular GSH content and activity of its related enzymes. In some resistant cell lines, an increase in intracellular GSH [29,30] and in the GST level of expression [31] have been reported.

It has been shown that GSTs, and especially the Pi isoform, are directly involved in the conjugation of cisplatin to GSH [32]. In our cell model, GSTs activity levels are not significantly different between the two cell lines (25.6 ± 2.8 nmol CDNB/min/mg protein and 27.8 ± 2.4 nmol CDNB/min/mg protein for HeLa and HeLa-GGT, respectively). Thus, the difference in the cytotoxicity due to carboplatin treatment can be directly correlated to the difference in GGT activity between HeLa and HeLa-GGT.

GGT activity is involved in intracellular GSH homeostasis, because cells overexpressing GGT can use extracellular GSH as a source of cys for the intracellular synthesis of the tripeptide [6]. We show that challenge of HeLa cells with cisplatin or carboplatin results in the induction of GGT and GCL expression, which is associated with an increase of the intracellular GSH level. Thus, an early response of HeLa cells to platinum drugs is an increased activity of GSH related enzymes, suggesting their important role in cellular defense. We have described recently a new extracellular cisplatin-detoxification mechanism that involves GSH and leads to resistance. In the presence of GGT, exogenous GSH is cleaved and the resulting product cysgly, forms adducts with cisplatin [14]. cysgly reacts with cisplatin at a rate 10-fold higher than that of GSH. Thus, GGT accelerates the formation of the adduct, and HeLa-GGT cells are more resistant to cisplatin than HeLa cells.

Using purified GGT from HeLa-GGT cells, we presently demonstrated that in the presence of GSH, the formation of the cysgly–cisplatin adduct depends solely on the catalytic activity of GGT. The fact that this extracellular mechanism could play an important role in the cellular response to cisplatin was suggested also by Paolicchi *et al*. [33], who showed that the thiols composition (GSH, glucys and GSSG content) of the extracellular medium depends on the activity of GGT, and that this thiols composition affects cisplatin cytotoxicity towards melanoma cells.

In a recent study, it has been shown that in HK-2 cells (a human cell line derived from the proximal convoluted tubule of the kidney), extracellular GSH decreases the antiproliferative effects of cisplatin only upon its hydrolysis by GGT, thereby supporting the hypothesis that the extracellular metabolism of GSH by GGT plays a role in modulating cisplatin nephrotoxicity [34]. Furthermore, it

was shown that 2 hr ''precomplexing'' of cisplatin with GSH in the presence of GGT, or directly with the GSH catabolite cysgly, decreased the antiproliferative effect of cisplatin and drug-induced DNA platination to a greater extent than ''precomplexing'' with GSH alone [34]. Thus, the mechanism of extracellular detoxification of cisplatin by the GGT-dependent GSH catabolism seems to be relevant in different cellular models.

We have also found that the level of GGT affects also the resistance to carboplatin. However, carboplatin did not form adduct neither in the presence of GSH or cysgly (not shown), nor in the presence of GGT and GSH. We propose that, in our model, the intracellular GSH level is responsible for the resistance to carboplatin, based on the association of this resistance with the cellular GSH level. The latter depended on the availability of thiols in the medium.

Interestingly, the increase in the viability of HeLa-GGT cells, cultured in the presence of GSH can be correlated to the higher GSH content of these cells. This increase in cell survival was observed even when cells were grown in the presence of a very low concentration of GSH (5 μM, equivalent to the concentration in human serum) [35].

Our results are also physiologically relevant as the concentrations of carboplatin presently used are consistent with those in clinical practice. The concentrations of carboplatin in our study varied from 25 to 150 μM (corresponding to a range from 9.3 to 55.7 mg/mL) and correspond to plasma ultrafiltrate concentrations measured 4 hr after drug administration in patients with advanced ovarian cancer treated with high-dose carboplatin chemotherapy [36].

In conclusion, our studies show that the level of GGT activity is involved directly in the cellular response to anticancer drug treatment. We propose that GGT could play a role in the resistance to cisplatin and carboplatin by extracellular and intracellular mechanisms, which depend on the reactivity of the platinum compound. This hypothesis is summarized in Fig. 6: cysgly is produced from exogenous GSH by GGT in the extracellular medium, and the chemically-reactive cisplatin forms non enzymatically an adduct with cysgly, thus detoxifying the drug and resulting in resistance. The less reactive carboplatin does not react to form adducts in the extracellular medium, but is hydrolyzed within the cell where its reactive form is detoxified by GSH (spontaneous reaction or catalyzed by GST). In this case, an increase of the GSH level or of the GSH turnover due to GGT-mediated supply of GSH precursors is determinant for the detoxification of carboplatin.

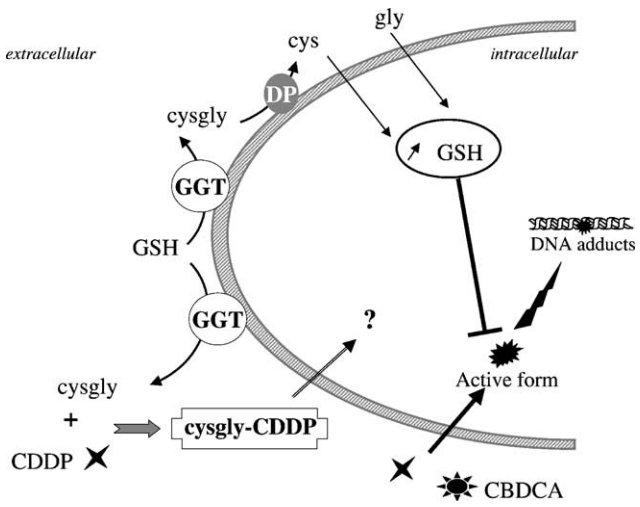

Fig. 6. Hypothetical mechanism of resistance to cisplatin and carboplatin in HeLa cells overexpressing GGT. CDDP: cisplatin, CBDCA: carboplatin, GSH: glutathione, GGT: γ-glutamyltransferase, cysgly: cysteinylglycine, DP: dipeptidase, cys: cystine, gly: glycine.

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