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Cellular response to oxidative stress and ascorbic acid in melanoma cells overexpressing γ-glutamyltransferase

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ABSTRACT

The extracellular γ-glutamyltransferase-mediated metabolism of glutathione has been implicated in prooxidant events which may have impact on cellular functions including drug resistance. This study was performed in two GGT-transfected melanoma clones to explore the hypothesis that GGT expression in tumour cells is implicated in modulation of cell behaviour under stress conditions. Our results show that GGT-overexpression in melanoma cells was associated with resistance to oxidative stress produced by prooxidant agents such as hydrogen peroxide and ascorbic acid. In GGT-overexpressing cells, ability to tolerate oxidative stress was evidenced by the presence of a moderate level of ROS and lack of DNA damage response following treatment with H_2O_2. Cellular response to oxidative stress induced by ascorbic acid was detectable only in the clone with low GGT activity which also exhibited an increased susceptibility to apoptosis. The increased resistance of the GGT-overexpressing clone was not related to intracellular GSH content but rather to the increased expression of catalase and to a reduced efficiency of iron-mediated formation of toxic free radicals. Taken together, these findings are consistent with a contribution of GGT in the mechanisms of drug resistance, because induction of oxidative stress is a relevant event in the apoptotic response to cytotoxic agents.

1. Introduction

γ-Glutamyltransferase (GGT), an ecto-enzyme over-expressed in a number of tumour cell types, is involved in cellular homeostasis of the major antioxidant tripeptide, glutathione (GSH). GGT catalyses the first step in the hydrolysis of extracellular GSH, ultimately allowing the recovery of cysteine, and for this reason it has been traditionally regarded as a major factor in the reconstitution of cellular antioxidant defences. Indeed, several studies have been devoted to the relationship between GGT expression and maintainance of cellular levels of GSH, which plays an important role in cancer resistance to cytotoxic therapy.[1] However, recent evidence supports a complex role of GGT in modulation of redox equilibria, with pathophysiological effects on both intracellular functions and extracellular microenvironment.[2] We have proposed that some of these effects may be implicated in tumour progression and drug resistance.[3] The persistent, low-level oxidative stress associated with GGT expression[4] might in fact stimulate the induction in cancer cells of protective systems involved in detoxification and/or resistance to cytotoxic agents.

To further explore the hypothesis that GGT (over)expression may provide cancer cells with survival advantages

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under stress conditions, in the present study we have investigated the cellular response of two human melanoma cell clones, characterised by a marked difference in GGT expression, to two agents affecting cellular redox status, hydrogen peroxide (H_2O_2) and ascorbic acid (AA). The results provide evidence that GGT overexpression in melanoma cells is associated with increased resistance to oxidative stress and AA-induced apoptosis. This effect is unrelated to cellular content of GSH, but rather appears to depend on the induction of protective (antioxidant, antiapoptotic) mechanisms.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell lines and culture conditions

Two human melanoma cell clones expressing different GGT activity and obtained as previously described[5] were used. Briefly, the c21/GGT clone and the c21/basal clone expressing high (90.78 ± 3.40 mU/mg of cellular protein) and low (0.34 ± 0.13 mU/mg of cellular protein) GGT activity respectively, were obtained by stable transfection of low-expressing GGT activity Me665/2/21 clone (c21) with the full-length cDNA of human GGT. Cells were routinely grown in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine (L-Gln) and 0.5 mg/ml G418 (Gibco), at 37 °C in a 5%/95% CO_2/air atmosphere.

2.3. Determination of GGT activity

Confluent cell monolayers were harvested with hypotonic lysis buffer (10 mM Tris–HCl, pH 7.8) and disrupted by a tight-fitting glass–glass Dounce homogeniser (30 strokes, 4 °C). Determination of GGT activity was performed according to Huseby and Strømme[6] using γ-glutamyl-*p*-nitroanilide as substrate and glycyl-glycine as transpeptidation acceptor. The amounts of *p*-nitroaniline formed were measured by reading the absorbance at 405 nm and using a molar extinction coefficient of 9200 mol/L· cm. One unit of GGT activity was defined as 1 μmol of substrate transformed/ml/min. The results were expressed as mU/mg protein.

2.4. Drugs and treatment conditions

Experiments with hydrogen peroxide (H_2O_2, 3% solution in water; OLCELLI Farmaceutici, Italy) and ascorbic acid (AA, 1000 mg/5 ml; S.A.L.F., Italy) were performed by incubating cells for 2 h at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum. In a separate set of experiments, incubations with AA were performed in the presence of glutathione (GSH, 0.5 mmol/L) and glycyl-glycine (glygly, 20 mmol/L), in order to achieve full activation of GGT.[6] Other experiments were performed in the presence of the iron chelators deferoxamine mesylate (DFO; 500 μM) and 2,2′-bipyridyl (BIP; 500 μM) and the antioxidant catalase (200 U/ml). Other experimental details are given in the legends to individual figures.

2.5. Cell viability assay

Cell sensitivity to hydrogen peroxide and ascorbic acid was determined by growth inhibition assay. Briefly, cells were seeded in 12-well plates (50,000 cells/well), 24 h before experiments. Cells were exposed to the drugs for 2 h and then washed, medium was changed, and cultures were grown up to 72 h.

After 72 h, adherent cells were trypsinised and counted by a cell counter (Coulter Electronics, Luton, UK). IC_{50} values, derived from dose-response curves, were defined as drug concentrations required for 50% inhibition of cell growth.

2.6. Reactive oxygen species (ROS) determination

Intracellular production of ROS was determined using the H_2O_2 sensitive compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Invitrogen, USA) as previously described.[7]

This method measures the formation of intracellular ROS. After diffusion into viable cells, DCFH-DA is converted by intracellular esterase to 2′,7′-dichlorofluorescin (DCFH) which is able to react quantitatively with reactive oxygen species within the cell thus producing the fluorescent dye 2′,7′-dichlorofluorescin (DCF). The latter remains trapped within the cells and can be measured as an index of intracellular ROS production.

Twenty-four hours after a 2 h treatment with H_2O_2 or ascorbic acid, cells were incubated with DCFH-DA (10 μmol/L; dissolved in DMSO), at 37 °C for 20 min. Culture medium was then removed, adherent and floating cells were washed twice with phosphate-buffered saline (PBS) and then collected. DCF fluorescence was detected by a flow cytometer (Becton Dickinson); for each sample 10,000 events were collected.

2.7. Cell cycle analysis

For cell cycle analysis, 24 h after drug treatments cells were washed, fixed in ice-cold 70% ethanol, and stored at −20 °C. Subsequently, samples were rehydratated with PBS and cellular DNA was stained with 10 μg/ml propidium iodide in PBS, containing RNase A (66 U/ml). Cell cycle distribution was determined by flow cytometry, and data were analysed by Cell Quest® software; for each sample 40,000 events were collected.

2.8. Western blot analysis

Cells were rinsed twice with ice-cold PBS supplemented with 0.1 mM sodium orthovanadate, and then lysed in hot sample buffer.[8] After determination of protein concentration, whole-cell extracts were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Immunoreactive bands were revealed by enhanced chemiluminescence detection (Amersham Biosciences, Rockford, IL) using anti-Bcl-2 (Dako,

Denmark), anti-Bax and anti-caspase 3 (BD Pharmingen, USA), anti-γ-H_2AX (Upstate Biotechnology, USA), anti-RPA-2 (NeoMarker, USA), anti-cytochrome C (BD Pharmingen), anti-phospho-p53(Ser[15]) (Cell Signaling Technology, USA) and anti-actin (Sigma) antibodies.

2.9. Determination of apoptosis

Apoptosis was determined by TUNEL assay 72 h after 2 h exposure to treatments. Treated cells were fixed in 4% paraformaldehyde (RT, 60 min), washed and resuspended in ice-cold PBS. The *in situ* cell death detection kit (Roche, Germany) was used according to the manufacturer's instructions, and samples were analysed by flow cytometry.

2.10. Determination of cytochrome c release from mitochondria

Melanoma cells were exposed to equitoxic H_2O_2 (100 μM and 4.6 mM for c21/basal and c21/GGT, respectively) or ascorbic acid concentrations (0.03 mM and 0.06 mM for c21/basal and c21/GGT, respectively) for 2 h. Floating and adherent cells were harvested and cytosolic extracts were prepared as described.[9] Briefly, cells were disrupted by a Dounce homogeniser (30 strokes) in ice-cold lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.5 mM phenyl-methyl-sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml trypsin inhibitor), then samples were centrifuged at 20,000 *g* for 20 min. Supernatants were stored at −70 °C until gel electrophoresis. Cytosolic protein extracts were run on a 15% SDS-PAGE gel and processed for Western blot analysis as described above.

2.11. Determination of catalase activity

The Amplex Red Catalase Assay Kit, used for this determination, was purchased from Molecular Probes (USA). In this assay, catalase first reacts with hydrogen peroxide to produce water and oxygen, then the Amplex Red reagent reacts with any unreacted H_2O_2 in the presence of horseradish peroxidase (HRP) to produce the highly fluorescent oxidation product, resorufin.

Catalase activity was determined 24 h after cell treatments. Briefly, cells were rinsed twice with ice-cold PBS supplemented with 0.1 mM sodium orthovanadate and then lysed in hot sample buffer.[8] Samples were then used to evaluate spectrophotometrically (550 nm) catalase activity according to manufacturer's instructions.

2.12. Other determinations

Protein content was determinated by the method of Bradford (Bio-Rad protein assay). Glutathione peroxidase activity was determined as described.[10] Glutathione (GSH) and glutathione disulfide (GSSG) were determined by enzymatic/spectrophotometric method.[5]

The reported values represent the mean ± standard deviation (SD) of at least three independent experiments. Statistical significance was assessed by ANOVA.

3. Results

3.1. Biochemical characterisation of cell clones

The study was performed on two human melanoma clones, characterised by a marked difference in GGT activity (Table 1), the c21/GGT clone exhibiting a high GGT activity (90.8 ± 3.4 mU/mg protein), in contrast to the low activity of the c21/basal clone (approx. 180 times lower). In spite of the substantial difference in GGT activity and expression (not shown), the GSH content was marginally higher in the c21/basal clone. Interestingly, the content of GSSG was appreciably increased in the GGT-overexpressing clone and the increased GSSG/GSH ratio supports conditions of oxidative stress. Cell doubling time was not modified by transfection procedures, and was approximately 24 h in both clones.

3.2. Cell response to H_2O_2-induced oxidative stress

We have investigated whether the overexpression of GGT could influence the antiproliferative activity of hydrogen peroxide. The effects of H_2O_2 were investigated both in standard cell culture conditions and in medium supplemented with GSH (500 μM) and glycyl-glycine (20 mM), in order to achieve full activation of GGT.[6] As shown in Fig. 1, when cells were exposed for 2 h to hydrogen peroxide under standard conditions, a different response was observed, with an IC_{50} value for c21/GGT cells (2.3 mM) about 50 times higher than that observed for c21/basal cells (0.05 mM). Similar differences were observed when clones were exposed to H_2O_2 in conditions of full GGT activation (data not shown).

On the basis of these results, two equitoxic hydrogen peroxide concentrations (4.6 mM and 100 μM for c21/GGT and c21/basal clone, respectively, causing approx. 70% inhibition of cell growth in standard culture medium) were chosen for subsequent experiments.

3.3. Effects of H_2O_2 on intracellular ROS production

Analysis of ROS production was performed 24 h after the end of H_2O_2 treatment. As shown in Fig. 2, in spite of the higher concentration of H_2O_2 applied, in the c21/GGT clone the extent of intracellular ROS production was lower than in c21/basal cells.

Table 1 – Melanoma clones characterisation

	c21/GGT	c21/basal
GGT activity (mU/mg protein)	90.78 ± 3.4	0.344 ± 0.13
GSH content (nmol GSH eq./mg protein)	51.25 ± 10.75	67.56 ± 8.6
GSSG content	1.05 ± 0.12	0.33 ± 0.32
GSSG/GSH ratio (%)	2.05	0.49
Doubling time (h)	24 ± 0.8	24 ± 1.2

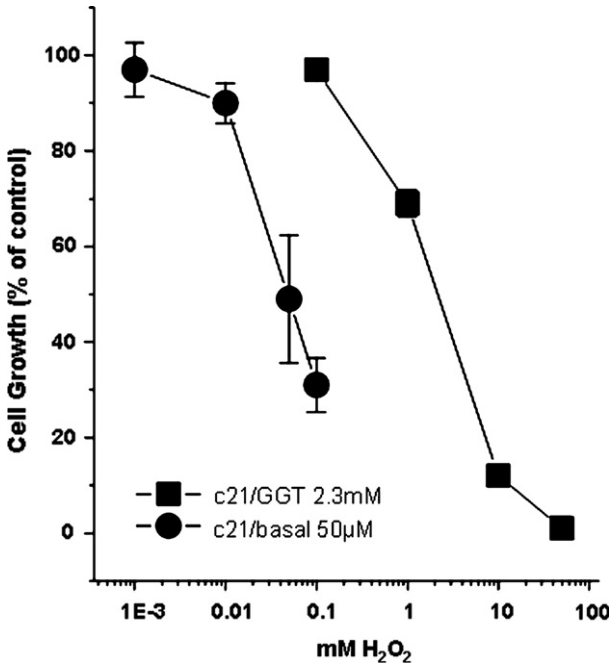

Fig. 1 – Cytotoxic effect of H_2O_2 on c21/GGT and c21/basal clone. Cells were treated for 2 h with H_2O_2 and cell viability was evaluated 72 h after the treatment by cell counting. Data are the mean of three independent experiments.

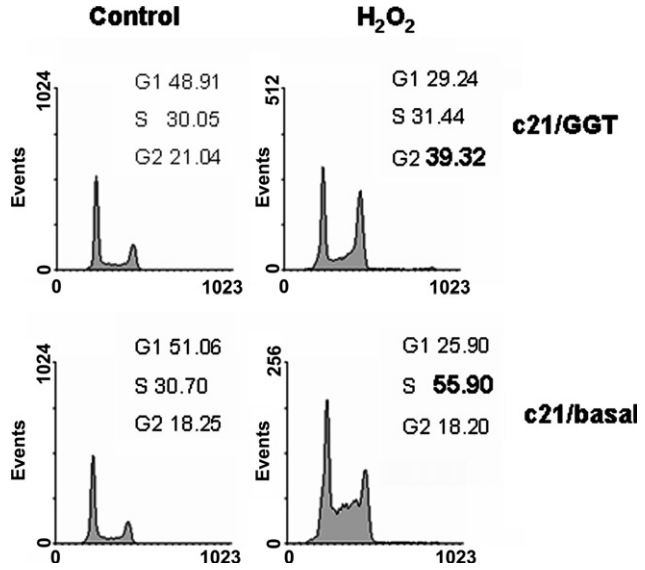

Fig. 3 – Cell cycle distribution after H_2O_2 exposure. Cells treated with the same equitoxic concentrations of H_2O_2 used for ROS induction analyses were examined for cell cycle distribution 24 h after the end of treatment. The percentages of cells in G1, S or G2 phases are reported in each histogram.

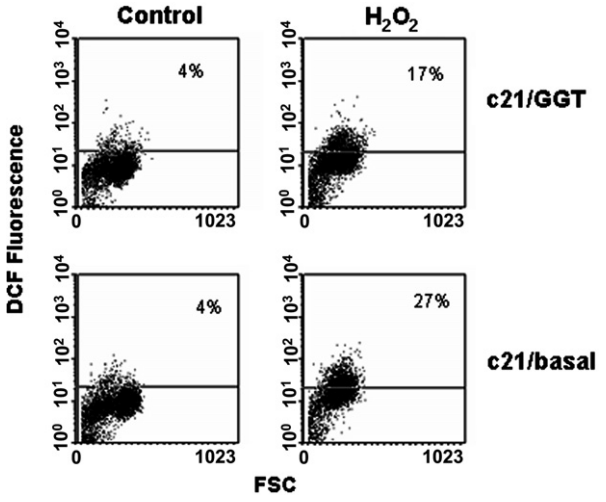

Fig. 2 – ROS induction 24 h after a 2 h H_2O_2 exposure. Cells treated with H_2O_2 (4.6 mM and 100 μM for c21/GGT and c21/basal clone, respectively) were incubated with 10 μmol/L DCFH-DA. The DCF fluorescence was detected by FACScan flow cytofluorimeter. One representative experiment out of three was reported. The percentages of DCF-positive cells are reported in each dot plot.

3.4. *Effects of H_2O_2 on cell cycle*

As shown in Fig. 3, equitoxic concentrations of H_2O_2 caused different effects on cell cycle distribution in the two clones. 24 h after a 2 h-exposure to H_2O_2, an accumulation in G2 phase was observed for c21/GGT cells, while the c21/basal clone showed an arrest in S phase concomitant with a decreased percentage of cells in G1 phase.

To elucidate the reasons for the differences observed, we examined the activation of three selected proteins involved in DNA damage/repair response, i.e. histone γ–H_2AX, phospho-p53 (Ser^{15}) and RPA-2. As assessed by western blot analysis (Fig. 4), exposure to equitoxic concentrations of H_2O_2 induced a higher induction of γ–H_2AX phosphorylation and phospho-p53 (Ser^{15}) in c21/basal than in c21/GGT cells, whereas RPA-2 was not activated in either clones.

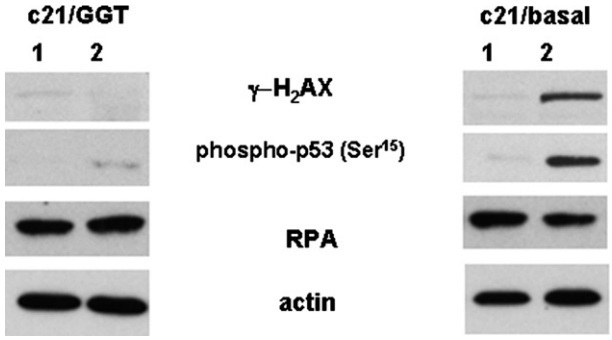

Fig. 4 – Induction of DNA damage response in c21/GGT and c21/basal cells exposed to equitoxic H_2O_2 concentration for 2 h. After 24 h from the end of treatment, cells were lysed and processed for Western blotting. 1 = control, 2 = H_2O_2 treatment (4.6 mM for c21/GGT and 100 μM for c21/basal clone). Loading control is shown by actin.

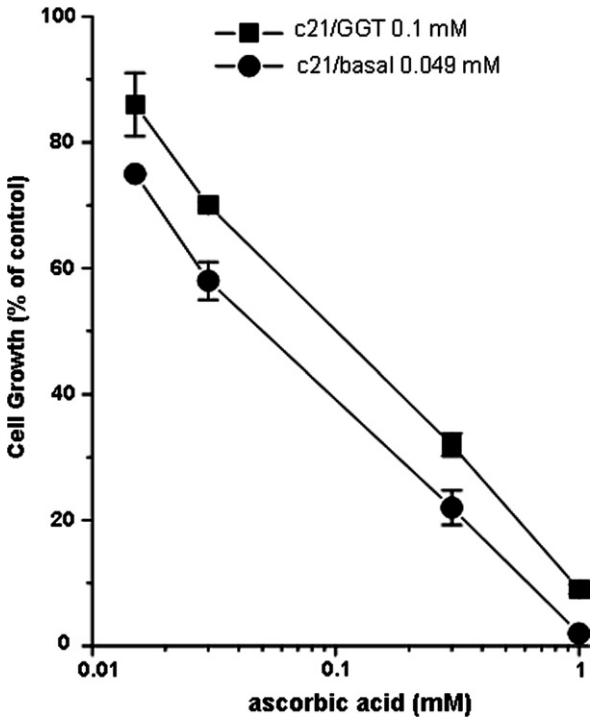

Fig. 5 – Antiproliferative effect of ascorbic acid. Dose-dependent effects 72 h after a 2 h ascorbic acid treatment of c21/GGT and c21/basal cells, in GGT activating condition. Data are the mean of three independent experiments.

3.5. *Effects of ascorbic acid on cell proliferation*

Exposure to AA for 2 h was able to exert an antiproliferative effect, which was two times higher in c21/basal than in c21/GGT cells (Fig. 5). Again, the differences observed between the two clones were similar in standard culture conditions and in the medium enriched with GSH and glycyl-glycine to activate GGT, even if in the latter condition AA citotoxicity was higher (Table 2). On the basis of these results, two equitoxic concentrations of AA (0.06 mM and 0.03 mM for c21/GGT and c21/basal, respectively, causing approximately 30% inhibition of cell growth in GGT activating condition) were chosen for subsequent experiments.

3.6. *Iron-mediated antiproliferative effects of H_2O_2 and ascorbic acid*

The involvement of redox-active iron in oxidative stress processes is a well established mechanism.[11] To explore the possible role of iron in H_2O_2 and AA cytotoxicity, we evaluated the cellular response to these oxidative agents after lowering extra- and/or intracellular free (ionic) iron concentrations. In order to remove extracellular iron, incubation mixtures were pre-treated with the iron chelator DFO, 1 h at 37 °C, before cell treatments. As shown in Fig. 6A-B, pre-treatment with DFO resulted in a reduced antiproliferative activity of H_2O_2 and AA in both clones. Interestingly, when the same incubation mixtures were pre-treated with bipyridyl, an iron chelator capable of entering the cell and chelating both extra- and intracellular free iron,[12] the antiproliferative effect of H_2O_2 and AA was almost completely prevented (Fig. 7A-B). The crucial role played by redox-active iron was further confirmed in experiments carried out at equimolar concentrations of AA. Indeed, in the presence of bipyridyl the sensitivity of c21/basal cells to AA was markedly reduced, down to a value similar to that observed in c21/GGT cells (from 65% to 40% dead cells, Fig. 8A).

Table 2 – Comparison of sensitivity of c21/GGT and c21/basal cells to ascorbic acid in standard culture medium and in GGT activating condition

	IC_{50} (mM)	
	c21/GGT	c21/basal
Standard culture medium	0.4 ± 0.06	0.2 ± 0.07
GGT activating condition	0.1 ± 0.05	0.049 ± 0.001

3.7. *Suppression of AA antiproliferative effects by exogenous catalase*

Further experiments were performed to assess the involvement of hydrogen peroxide in AA antiproliferative effect. As shown in Fig. 8B, after pre-treatment with catalase (200 U/ml, 1 h, 37 °C) sensitivity to AA was significantly reduced in both clones, and the difference between clones was abolished.

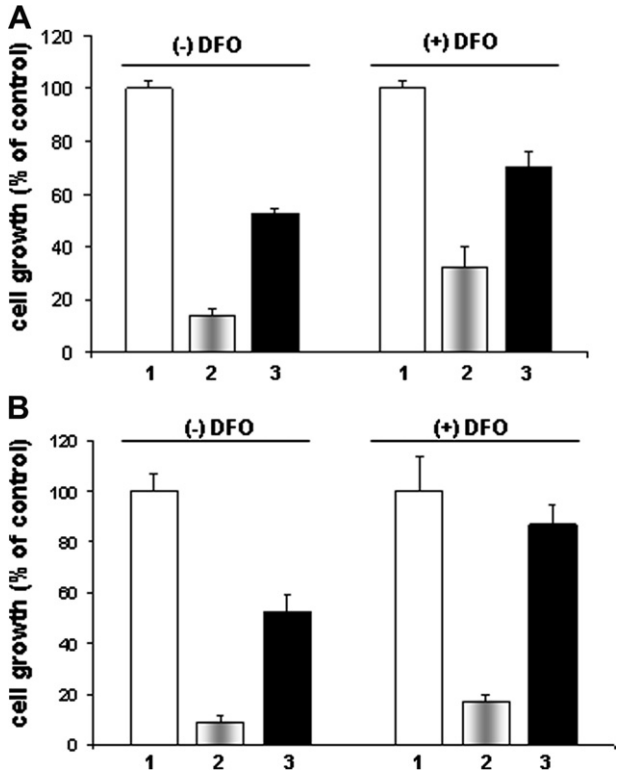

Fig. 6 – Cellular sensitivity of c21/GGT (A) and c21/basal (B) cells to H_2O_2 and ascorbic acid in the presence or absence of DFO. Cells were treated for 2 h with a medium pre-incubated with ascorbic acid or H_2O_2 in the presence/absence of DFO (500 μM; 1 h, 37 °C). 1 = control; 2 = H_2O_2 (4.6 mM and 100 μM for c21/GGT and c21/basal, respectively); 3 = AA (0.06 mM and 0.03 mM for c21/GGT and c21/basal, respectively).

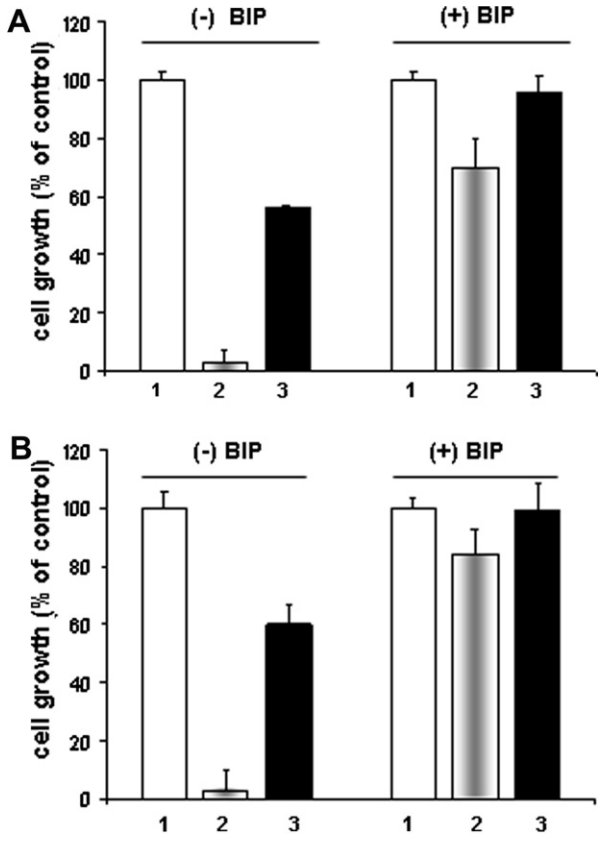

Fig. 7 – Cellular sensitivity of c21/GGT (A) and c21/basal (B) cells to H_2O_2 and ascorbic acid in the presence or absence of bipyridyl (BIP). Cells were treated for 2 h with a medium pre-incubated with ascorbic acid or H_2O_2 in the presence/absence of bipyridyl (500 μM; 1 h, 37 °C). 1 = control; 2 = H_2O_2 (4.6 mM and 100 μM for c21/GGT and c21/basal, respectively); 3 = AA (0.06 mM and 0.03 mM for c21/GGT and c21/basal, respectively).

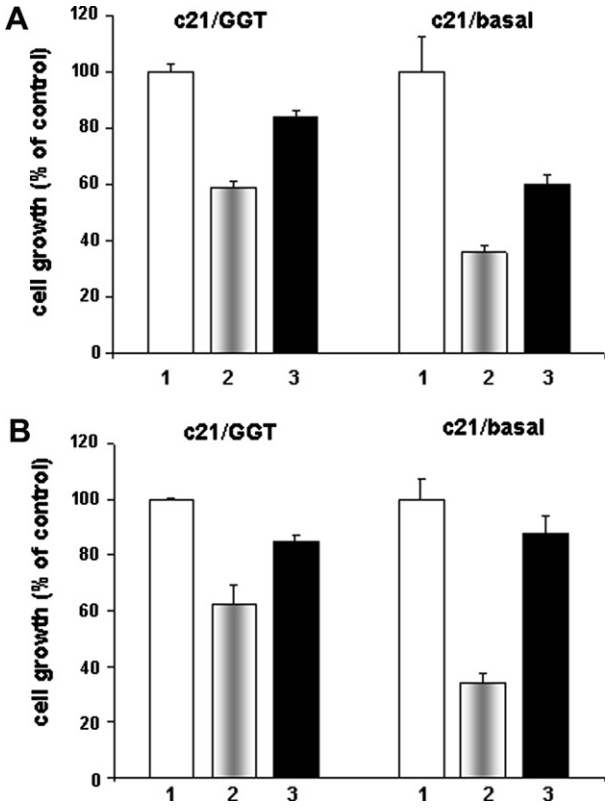

Fig. 8 – Cellular sensitivity of c21/GGT and c21/basal clone to ascorbic acid 0.06 mM in the presence or absence of bipyridyl (BIP) (A) or in the presence or absence of catalase 200 U/ml (B). (A) Cells were treated for 2 h with a medium pre-incubated with ascorbic acid (equimolar concentration, 0.06 mM) in the presence/absence of bipyridyl (500 μM; 1 h, 37 °C). 1 = control; 2 = AA 0.06 mM; 3 = AA (0.06 mM in the presence of bipyridyl 500 μM). (B) Cells were treated with a medium containing ascorbic acid (equimolar concentration, 0.06 mM) or ascorbic acid and catalase 200 U/ml. 1 = control; 2 = AA 0.06 mM and 3 = AA (0.06 mM in the presence of catalase 200 U/ml).

3.8. *AA treatment causes induction of apoptosis and ROS production*

Fig. 9 shows the results obtained with TUNEL assay 72 h after treating clones for 2 h with equitoxic concentration of AA. Ascorbic acid was able to cause a marked apoptotic cell death in c21/basal clone (27% TUNEL positive cells) while no appreciable effect was detectable in c21/GGT cells (3% TUNEL positive cells). Deeper analysis of the apoptotic behaviour of the two clones showed that cytochrome c release was induced by AA treatment in both clones (Fig. 10A), whereas cleavage of CPP32 was detectable only in c21/basal clone (Fig. 10B). No differences were observed either for Bax or Bcl-2 protein levels (Fig. 10B).

Fig. 11 shows intracellular ROS induction in the two clones under the same experimental conditions used in TUNEL assay. DCF fluorescence induced by AA in c21/basal was at least six times higher than in c21/GGT cells.

3.9. *AA treatment activates DNA damage response and cell cycle arrest*

DNA damage and cell cycle progression were investigated 24 h after AA treatment. The results showed an activation of DNA-damage responsive histone γ-H_2AX and phospho-p53 (Ser^{15}), which was more marked in c21/basal cells (Fig. 12). Cell cycle analysis indicated an accumulation of both clones in S phase (Table 3), suggesting the activation of a DNA damage checkpoint.

3.10. *Constitutive catalase activity in c21/GGT cells*

In order to elucidate the cellular basis for lower presence of ROS after H_2O_2 and AA treatment in c21/GGT cells, two major cellular enzymes involved in hydrogen peroxide catabolism, catalase and glutathione peroxidase, were investigated. Basal catalase activity was actually two times higher in c21/GGT than in c21/basal cells (Fig. 13). This difference reflected the expression of the enzyme as documented by Western blot analysis. Treatment with H_2O_2 induced a further increase of catalase activity, more evident in c21/basal cells. A marked induction of catalase activity (56%) was caused by AA treatment only in c21/basal cells. In all conditions studied, catalase activity was anyway higher in c21/GGT cells. In both

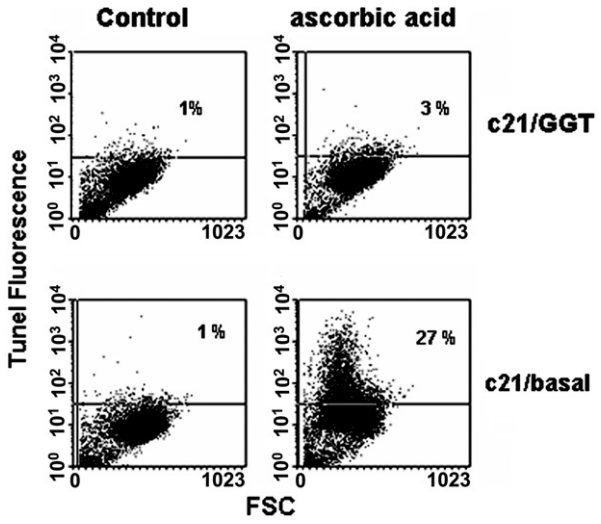

Fig. 9 – Apoptosis induced by ascorbic acid. Cells were exposed for 2 h to 0.06 mM and 0.03 mM ascorbic acid corresponding to equitoxic value in c21/GGT and c21/basal cells, respectively. Apoptosis was detected by TUNEL assay 72 h after the end of the treatment and determined by FACS analysis. Numbers in the dot-plots indicate the percentage of tunel positive cells.

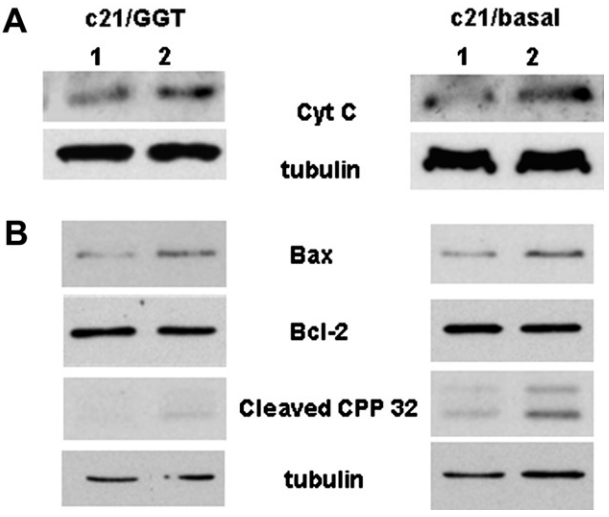

Fig. 10 – Effect of ascorbic acid treatment on the apoptotic pathway. (A) Effect of ascorbic acid on cytochrome c release. The two cell lines were exposed to equitoxic concentration of ascorbic acid and the cytosolic extracts were prepared 24 h after the drug exposure. (B) Bax, Bcl-2 and CPP32 cleavage levels. Total cellular extracts were obtained 24 h after cell treatment. Tubulin is shown as a control for protein loading. Lane 1: control; lane 2: ascorbic acid.

clones, no substantial modifications were found in catalase expression after treatments.

With regard to glutathione peroxidase basal activity, no differences were found between the two clones (8.5 ± 1.1 and 8.4 ± 0.8 mU/mg of protein for c21/GGT and c21/basal cells, respectively).

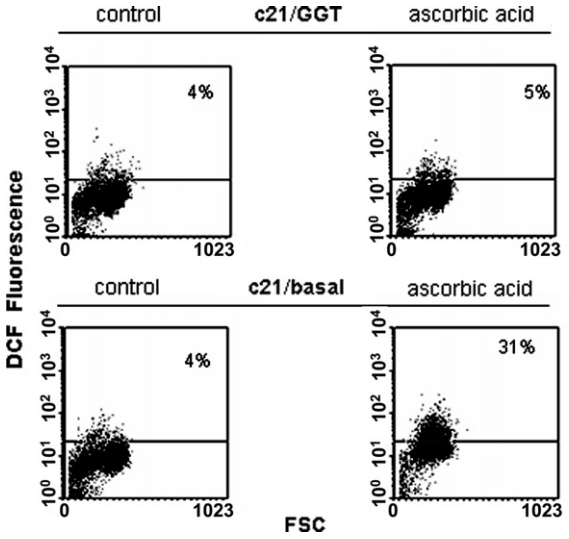

Fig. 11 – ROS induction by ascorbic acid. Cells were exposed for 2 h to 0.06 mM and 0.03 mM ascorbic acid for c21/GGT and c21/basal cells, respectively. After 24 h, ROS levels were detected by DCFH-DA assay and determined by FACS analysis.

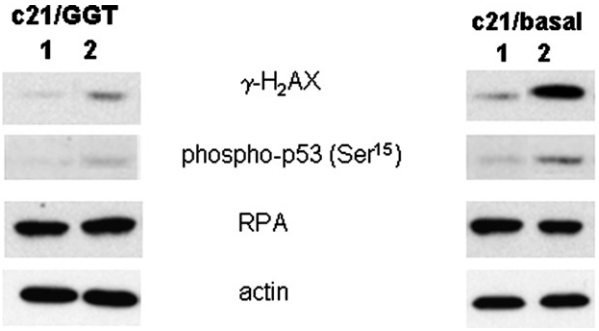

Fig. 12 – Induction of DNA damage response in c21/GGT and c21/basal cells exposed to equitoxic ascorbic acid concentration for 2 h. Protein expression of phospho-p53 (Ser^{15}), RPA, γ-H_2AX, and actin (loading control) after ascorbic acid treatment. 1 = Control, 2 = ascorbic acid treatment (0.06 mM and 0.03 mM for c21/GGT and c21/basal respectively).

4. Discussion

In the melanoma cell system used in the present study, we found that overexpression of GGT was associated with a reduced sensitivity to oxidative stress caused by H_2O_2, as well as to cytotoxic effects of ascorbic acid. The GGT-overexpressing c21/GGT cells were markedly more resistant to H_2O_2 and AA treatments, approx. 50- and 2-fold more than the c21/basal clone (Figs. 1 and 5). The ability of ascorbic acid to induce indirectly oxidant effects is a well established phenomenon, described in different cell types[13–15] and different culture conditions,[16,17] and likely related to AA interactions with trace levels of transition metal ions present in many incubation media. Thus, an increased resistance of c21/GGT clone to oxidative injury could explain both observations. Only moderate

Table 3 – Cell cycle distribution of c21/GGT and c21/basal cells after treatment with equitoxic concentration of ascorbic acid (one experiment representative of three is shown)

	c21/GGT			c21/basal		
	G1	S	G2	G1	S	G2
Control	52.3	28.04	19.66	47.69	29.22	23.1
Ascorbic acid	29.17	**48.46**	22.37	32.97	**53.82**	13.21

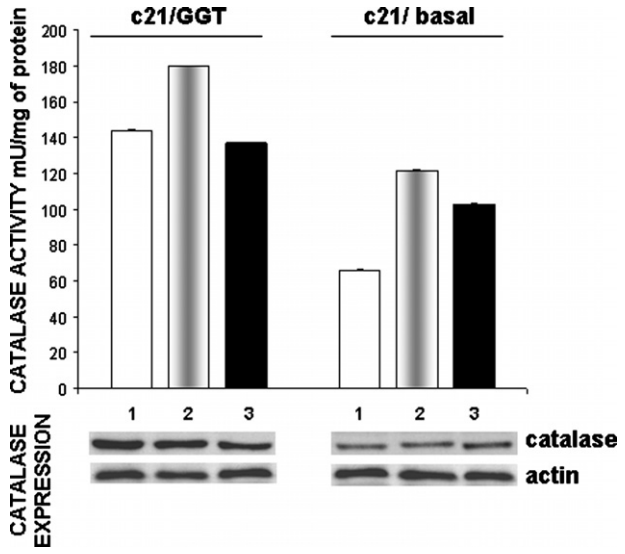

Fig. 13 – Catalase activity and expression. Cells were exposed for 2 h to H_2O_2 or to ascorbic acid, and catalase activity and expression were determined 24 h after treatment (see Materials and Methods for details). Actin is shown as loading control. One representative analysis out of three is shown. 1, control; 2, H_2O_2 (4.6 mM and 100 μM for c21/GGT and c21/basal cells, respectively); 3, Ascorbic Acid (0.06 mM and 0.03 mM for c21/GGT and c21/basal cells, respectively).

levels of ROS were indeed produced in c21/GGT cells following exposure to relatively high concentrations of H_2O_2 (Fig. 2), while paradoxically higher levels of ROS were produced in c21/basal clone (Fig. 9) exposed to substantially *lower* (equitoxic) H_2O_2 concentrations. Consistent with this finding, H_2O_2 treatment produced no DNA damage response in c21/GGT clone, i.e. neither histone γ–H_2AX phosphorylation nor S phase checkpoint activation, wherease a typical DNA damage response was found in c21/basal clone, with S phase cell accumulation and phosphorylation of both histone γ–H_2AX and p53 (Fig. 4).

The increased catalase expression and activity observed in untreated c21/GGT cells (two times higher as compared to c21/basal, Fig. 13) could account for the increased ability of this clone to tolerate oxidative stress, and likely reflects an adaptation phenomenon. In fact, GGT-mediated metabolism of extracellular GSH is known to produce prooxidant effects, including production of H_2O_2.[18] The occurrence of low but persistent oxidative stress in c21/GGT cells is consistent with a higher GSSG/GSH ratio (Table 1). It is likely that the ongoing GGT-mediated production of prooxidant can induce activation of protective pathways, including catalase expression. Anyway, a short-time exposure to cytotoxic concentrations of hydrogen peroxide or AA resulted in a significant induction of catalase activity, but not of its expression level. Thus it is conceivable that an acute oxidative stress causes an activation of the catalase already present in the cell, whereas a low persistent oxidative stress, present in the GGT-overexpressing cells as a consequence of GGT-mediated extracellular metabolism of GSH, results in modulation of protein expression (Fig. 13).

Ascorbic acid was used in this study as a prooxidant agent to explore possible pharmacological implications of the features acquired by the GGT-overexpressing phenotype, because AA may have a therapeutic potential in melanoma.[19,20] In spite of a comparable perturbation induced by AA on cell cycle with partial accumulation of cells in S-phase, the final outcome was substantially different in the two clones. In the more sensitive c21/basal clone, typical manifestations of oxidative stress were observed, including production of ROS (Fig. 11), DNA damage response (γ–H_2AX and p53 phosphorylation, Fig. 12) and activation of cell death pathways (cytochrome c release and caspase activation, Fig. 10). In contrast, following AA treatment the less responsive c21/GGT clone exhibited only marginal signs of genotoxic stress, with no evidence of apoptotic cell death. Such findings might be explained by the occurrence in c21/GGT clone of marginal, easily repairable DNA damage, and/or by a lack of recognition of DNA lesions. AA is known to induce prooxidant effects, and it was shown that the formation of H_2O_2 is a critical step in its cytotoxicity.[13–15] In agreement with this interpretation, the addition of exogenous catalase provided a substantial protection in our experiments (Fig. 8B). Therefore, the increased resistance of c21/GGT cells to AA more likely reflects their increased ability to detoxify H_2O_2 following upregulation of cellular catalase activity.

It is well established that prooxidant rather than antioxidant effects of AA depend on its interactions with transition metal ions, in particular iron.[21] Iron can also interact with H_2O_2 in the so-called Fenton reaction, leading to production of highly reactive and toxic hydroxyl radicals.[22] Indeed, data reported indicate that free, redox-active iron is implicated in the phenomena observed. When the iron-chelating agent DFO was added to incubation mixtures, attenuation of cytotoxicity of both H_2O_2 and AA was in fact observed (Fig. 6), and even stronger protection was achieved using 2,2′-bipyridyl, an agent able to permeate cells and hence to chelate both extracellular and intracellular free iron (Figs. 7 and 8A). Relevant to this point is the observation that, following exposure to an equimolar concentration (60 μM), the differential toxicity of AA between the two clones can be abolished by treating the c21/basal clone with 2,2′-bipyridyl. The lower sensitivity of the GGT-expressing clone could thus support a lower

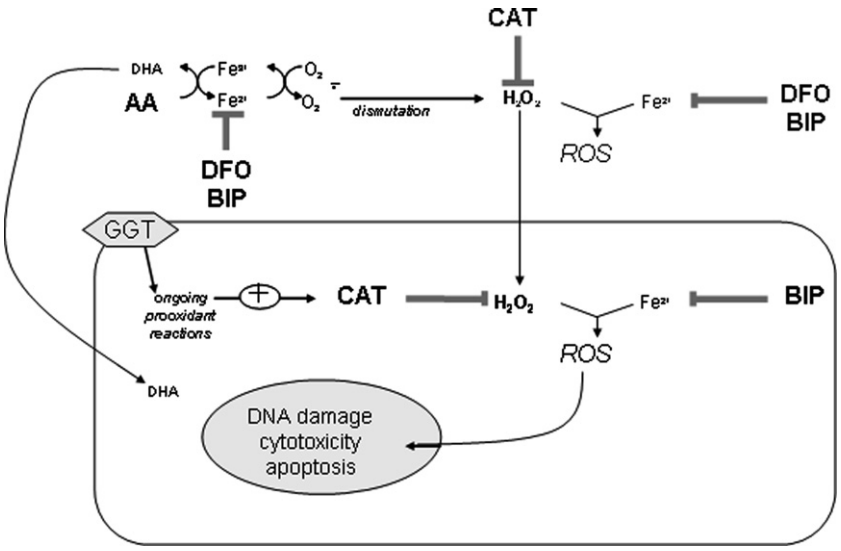

Fig. 14 – Scheme for the proposed mechanism of action of AA and H_2O_2 on the two melanoma clones and possible defence mechanisms. AA, ascorbic acid; BIP, bipyridyl; CAT, catalase; DFO, deferoxamine; DHA, dehydroascorbic acid.

content of intracellular free iron, required for the efficiency of redox-cycling processes, necessary to overdraw the inner oxidative burst. Indeed, free iron is known to participate in reaction resulting in free radical formation and oxidative stress leading to DNA damage and cell death. On the other hand, we have previously shown that iron-mediated oxidation of extracellular AA can favour its uptake by melanoma cells, and that this process is suppressed by iron chelation.[23] Protection offered by DFO and bipyridyl against AA effects may also depend on this phenomenon.

In conclusion, our study provides evidence that overexpression of GGT in melanoma cells is associated with increased resistance to oxidative stress depending on the induction of protective mechanisms against reactive oxygen species. Such a resistance to oxidative stress can explain the decreased susceptibility of GGT-expressing cells to effects of ascorbic acid. The marked resistance of GGT-overexpressing cells to oxidative stress can thus have important pharmacological implications because production of oxidative stress is a relevant event in the apoptotic response to several cytotoxic agents. Fig. 14 depicts our proposed mechanism of cellular stress induced by H_2O_2 or AA, and the possible defence mechanisms involved. Altogether, the results of the present study further support the implication of GGT as a factor in drug resistance of tumour cells.

Conflict of interest statement

None declared.

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REFERENCES

1. Pompella A, De Tata V, Paolicchi A, Zunino F. Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochem Pharmacol* 2006;**71**:231–8.
2. Paolicchi A, Dominici S, Pieri L, Maellaro E, Pompella A. Glutathione catabolism as a signaling mechanism. *Biochem Pharmacol* 2002;**64**:1027–35.
3. Pompella A, Corti A, Paolicchi A, Giommarelli C, Zunino F. gamma-Glutamyltransferase, redox regulation and cancer drug resistance. *Curr Opin Pharmacol* 2007;**7**:360–6.
4. Dominici S, Paolicchi A, Corti A, Maellaro E, Pompella A. Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyltransferase activity. *Methods Enzymol* 2005;**401**:484–501.
5. Franzini M, Corti A, Lorenzini E, et al. Modulation of cell growth and cisplatin sensitivity by membrane gamma-glutamyltransferase in melanoma cells. *Eur J Cancer* 2006;**42**:2623–30.
6. Huseby NE, Stromme JH. Practical points regarding routine determination of gamma-glutamyl transferase (gamma-GT) in serum with a kinetic method at 37 C. *Scand J Clin Lab Invest* 1974;**34**:357–63.
7. Li J, Tang Q, Li Y, et al. Role of oxidative stress in the apoptosis of hepatocellular carcinoma induced by combination of arsenic trioxide and ascorbic acid. *Acta Pharmacologica Sinica* 2006;**27**:1078–84.
8. Zuco V, Zanchi C, Cassinelli G, et al. Induction of apoptosis and stress response in ovarian carcinoma cell lines treated with ST1926, an atypical retinoid. *Cell Death Differ* 2004;**11**:280–9.
9. Milner AE, Palmer DH, Hodgkin EA, et al. Induction of apoptosis by chemotherapeutic drugs: the role of FADD in activation of

caspase-8 and synergy with death receptor ligands in ovarian carcinoma cells. *Cell Death Differ* 2002;**9**:287–300.
10. Paolicchi A, Lorenzini E, Perego P, et al. Extra-cellular thiol metabolism in clones of human metastatic melanoma with different gamma-glutamyl transpeptidase expression: implications for cell response to platinum-based drugs. *Int J Cancer* 2002;**97**:740–5.
11. Aust SD, Morehouse LA, Thomas CE. Role of metals in oxygen radical reactions. *J Free Radic Biol Med* 1985;**1**:3–25.
12. Konijn AM, Glickstein H, Vaisman B, Meyron-Holtz EG, Slotki ZI, Cabantchik ZI. The cellular labile iron pool and intracellular ferritin in K562 cells. *Blood* 1999;**94**:2128–34.
13. Peterkofsky B, Prather W. Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. *J Cell Physiol* 1977;**90**:61–70.
14. Duarte TL, Almeida GM, Jones GD. Investigation of the role of extracellular H_2O_2 and transition metal ions in the genotoxic action of ascorbic acid in cell culture models. *Toxicol Lett* 2007;**170**:57–65.
15. Park S, Han SS, Park CH, et al. Ascorbic acid induces apoptosis in acute myeloid leukemia cells via hydrogen peroxide-mediated mechanisms. *Int J Biochem Cell Biol* 1994;**36**:2180–95.
16. Clement MV, Ramalingam J, Long LH, Halliwell B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid. Redox Signal* 2001;**3**:157–63.
17. Sakagami H, Satoh K, Sugaya K, et al. Effect of the type of serum in the medium on sodium ascorbate-induced cytotoxicity. *Anticancer Res* 1996;**16**:1937–41.
18. Dominici S, Valentini M, Maellaro E, et al. Redox modulation of cell surface protein thiols in U937 lymphoma cells: the role of gamma-glutamyl transpeptidase-dependent H_2O_2 production and S-thiolation. *Free Rad Biol Med* 1999;**27**:623–35.
19. Lin SY, Lai WW, Chou CC, et al. Sodium ascorbate inhibits growth via the induction of cell cycle arrest and apoptosis in human malignant melanoma A375.S2 cells. *Melanoma Res* 2006;**16**:509–19.
20. Roomi MW, Ivanov V, Netke S, Kalinovsky T, Niedzwiecki A, Rath M. In vivo and in vitro antitumor effect of ascorbic acid, lysine, proline and green tea extract on human melanoma cell line A2058. *In Vivo* 2006;**20**:25–32.
21. Minotti G, Aust SD. Redox cycling of iron and lipid peroxidation. *Lipids* 1992;**27**:219–26.
22. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. Oxford: Oxford Science Publications; 1999.
23. Corti A, Raggi C, Franzini M, Paolicchi A, Pompella A, Casini AF. Plasma membrane gamma-glutamyltransferase activity facilitates the uptake of vitamin C in melanoma cells. *Free Radic Biol Med* 2004;**37**:1906–15.