Resistance to Cisplatin and Adriamycin is Associated with the Inhibition of Glutathione Efflux in MCF-7-derived Cells

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Abstract. *The impact of the anti-cancer drugs cisplatin (CDDP) and adriamycin (ADR) was investigated on sensitive and resistant MCF-7-derived human breast cancer cells. Cytotoxicity was evaluated by MTT assay, reactive oxygen species (ROS), apoptosis and necrosis by flow cytometry, glutathione (GSH) by HPLC, and Bcl-2, Bax and PARP expression by Western blot. A perturbation of ROS and intracellular GSH levels, and the enhancement of both apoptosis and necrosis were observed in sensitive cells. Transfected MCF-7 cells overexpressing the anti-apoptotic Bcl-2 protein, as well as MCF-7-derived vincristine-resistant cell line (Vcr-R) were resistant to both drugs. This resistance was clearly associated with an unaltered GSH level and with the inhibition of an early GSH efflux. Vcr-R cell resistance seemed to rely on a different mechanism, since it was found to be independent of Bcl-2 expression. Since Bcl-2 overexpression confers the strongest degree of resistance of MCF-7-derived cells, our observations further highlight Bcl-2 as a prime pharmacological target to sensitize cancer cells to chemotherapeutic agents.*

The main obstacles to effective treatments for human malignancies are the intrinsic and acquired resistance of tumor cells to chemotherapeutic drugs, as well as their inability to induce apoptotic cell death (1). The sensitivity of tumor cells to chemotherapy is critically determined by genes which regulate the apoptotic process among which is the Bcl-2 genes family (2). Its gene products are either anti-apoptotic like Bcl-2 or pro-apoptotic like Bax, and the ratio between these two subsets is an important determinant of sensitivity to cell death signals (3). Bcl-2 blocks the ability of reactive oxygen species (ROS) to mediate apoptosis (4) and its overexpression increases the intracellular level of the antioxidant glutathione (GSH) in various cell systems (5).

The glutathione buffer system modulates cell response to redox changes and GSH efflux often appears as an essential death regulator (6). Interestingly, it has been recently shown that murine melanoma cells with high metastatic potential overexpressed Bcl-2, showed high GSH content, no change in the GSH synthesis rate, but a decrease in GSH efflux (7). This suggests that Bcl-2 can increase metastatic cell resistance against oxidative stress by inhibiting GSH export and preserving intracellular GSH.

Based on these findings, the anti-apoptotic and anti-oxidant function of Bcl-2 was investigated, using different but related cell models: the sensitive breast cancer cell line MCF-7, its vincristine-resistant counterpart (Vcr-R), and MCF-7 transfected to overexpress or not Bcl-2 and respectively named Bcl-2 and Neo cells. This was carried out in the presence of two different anti-cancer drugs, adriamycin (ADR) and cisplatin (CDDP). Adriamycin is a topoisomerase II poison which has been reported to also induce oxidative stress (8), whereas cisplatin, a DNA alkylating agent, is known to cause cell death by mechanisms primary related to the formation of DNA adducts (9). The mechanism of action of the two drugs on the four cell models was compared by focusing in particular on the role of the GSH status in cell resistance.

Materials and Methods

Cell culture and transfection. Cells were maintained in RPMI-1640 medium (Eurobio) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10^4 UI/mL penicillin G, 10 mg/mL streptomycin and 25 μg/mL amphotericin B at 37˚C in a humidified atmosphere containing 5% CO_2. Stable transfection with the plasmids pSFFV-Neo and pSFFV-Bcl-2 (a generous gift of C.M. Rudin, University of Chicago) was performed using the TransIT®-LT1 transfection reagent according to manufacturer (Mirus). Clones were selected in medium containing 0.8 mg/mL G418. Both Neo and Bcl-2 clones were screened by Western blotting for Bcl-2 expression.

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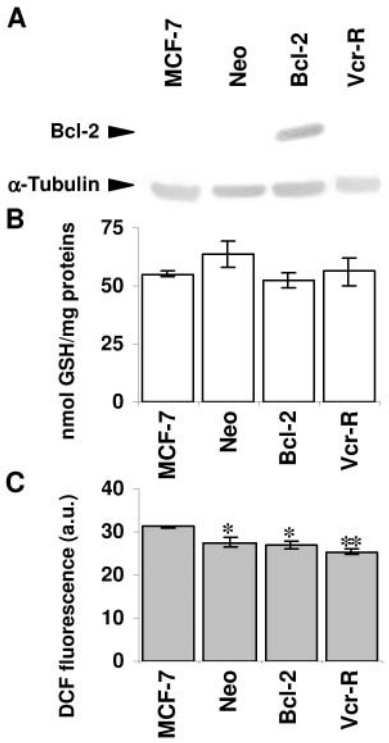

Figure 1. *Bcl-2 expression and basal GSH and ROS levels in MCF-7-derived cells. (A) Protein level of Bcl-2 in MCF-7, Neo, Bcl-2 and Vcr-R cells. Bcl-2 detected on total protein extracts by Western blot analysis, with α-tubulin used as reference. The immunoblot shown is from one experiment representative of three that gave similar results. (B) GSH level and (C) intracellular ROS level evaluated by flow cytometry as DCF fluorescence after a 24 h-culture in the four cell lines. Data are means ± SD (n=3), *p<0.05, **p<0.01 with respect to control (MCF-7).*

Treatments. In all experiments, cells were plated at a density of 15000 cells/cm^2. Twenty-four h after seeding, cells were treated with 5 μM ADR (Pharmacia) or 50 μM CDDP (Sigma) for the times indicated in figures legend. Controls were run in the presence of solvent alone (0.5% dimethyl sulfoxide, DMSO).

Western blotting. The cell pellet (floating and scraped adherent cells) lysis, homogenate preparation, protein electrophoresis and Western blot were performed as previously described (10). Blots were first probed with anti-Bcl-2 (1:1000) (C-2; Santa Cruz), anti-Bax (1:4000) (B-9; SantaCruz), anti-PARP (1:5000) (Roche), or anti-α-Tubulin (1:5000) (DM 1A; Sigma) antibodies, the latter used as reference. HRP-conjugated secondary antibodies (KPL) were used to detect immunoreactive bands using West Pico SuperSignal chemiluminescence reagent (Pierce).

Glutathione quantification. Dishes were kept on ice throughout the preparation. The medium was rapidly removed. Adherent cells were washed twice with ice-cold PBS and scraped in 10% perchloric acid to precipitate proteins. The cell suspension was centrifuged at 4˚C for 10 min at 12000 xg. Supernatant and pellet were frozen at –80˚C until appropriate quantifications. GSH and glutathione disulfide (GSSG) were quantified by a modified HPLC method based on Lenton *et al.* (11), using ortho-phtalaldehyde at 1.5 mM.

Glutathione efflux. Four, 8 or 12 h after ADR or CDDP intoxication, the culture medium was removed, cells were washed twice and incubated for 4 h with Krebs-Henseleit buffer. The buffer was then centrifuged and the supernatant was used to determine extracellular GSH whereas intracellular GSH was measured from adherent cells in plates as described above.

Flow cytometry analysis. Detection of cell death was determined by Annexin-V FITC staining (BD Biosciences), as previously described (12). ADR autofluorescence interferes with propidium iodide fluorescence, but a readjustment as recommended by Weinstein-Oppenheimer *et al.* (13) was applied to counteract it. For ROS detection, cells were incubated with 50 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) at 37˚C for 15 min. Total cells were then trypsin-harvested, resuspended in PBS and detected in the FL-1 (green) on a FACScan cytofluorometer (Becton-Dickinson).

Protein quantification. Perchloric acid-precipitated proteins were solubilized in one volume of 1N NaOH prior to protein concentration determination by the method of Lowry *et al.* (14) with bovin serum albumin used as standard.

Statistical analysis. Results are presented as mean ± SD and analyzed using a Student's *t*-test to assess statistical significance. A *p*-value of less than 0.05 was considered statistically significant.

Results

Overexpression of Bcl-2 and basal GSH and ROS levels. The effectiveness of the transfections was evaluated by Western blotting. The overexpression of the 26 kDa Bcl-2 protein was only observed in cells transfected with the pSFFV-Bcl-2 vector (Figure 1A).

Since Bcl-2 overexpression has been shown to play an anti-oxidant function related to an increase in GSH content in various cell lines (5), the basal level of the tripeptide was quantified in the different cell lines studied here. GSH level was nearly the same regardless of the cells (Figure 1B), and no GSSG was detected (data not shown). Furthermore, ROS levels, measured by flow cytometry, were the lowest in Vcr-R cells and were not significantly different between Bcl-2 and Neo cells, even if these levels were slightly lower than in MCF-7 (Figure 1C).

Cell death, ROS and intracellular GSH during ADR and CDDP treatments. We sought to characterize the effect of cytotoxic insults caused by ADR and CDDP cell death induction in MCF-7-derived cells. These treatments provoked an apoptosis and necrosis induction in MCF-7 and Neo cells (Table I). This induction was moderate in Vcr-R cells whereas overexpression of Bcl-2 efficiently protects cells against cell death (Table I).

Apoptosis can be initiated by the release of mitochondrial cytochrome c which event is controlled by the Bax/Bcl-2 ratio (15) prior to poly (ADP-ribose) polymerase (PARP) cleavage. No significant effect on the expression of Bax and

Table I. *Bcl-2 and Vcr-R cells are resistant to ADR and CDDP-treatment. Apoptosis and necrosis were quantified by Annexin-V flow cytometric assay after a 24 h-treatment. -, no induction.*

		Fold induction *vs.* control	
		Apoptosis	Necrosis
MCF-7	ADR	5	5
	CDDP	5	3
Neo	ADR	7.5	2
	CDDP	6.5	1.5
Bcl-2	ADR	-	-
	CDDP	-	-
Vcr-R	ADR	-	2
	CDDP	2	1.5

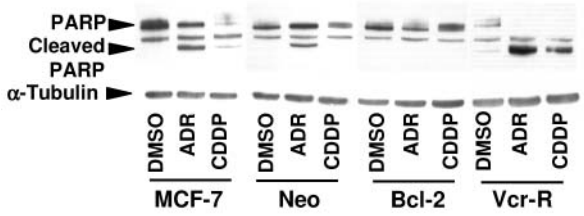

Figure 2. *PARP cleavage in the different cell lines after 48 h-treatment. Protein expression and PARP cleavage were determined by Western blot analysis. Representative results are one of two independent experiments.*

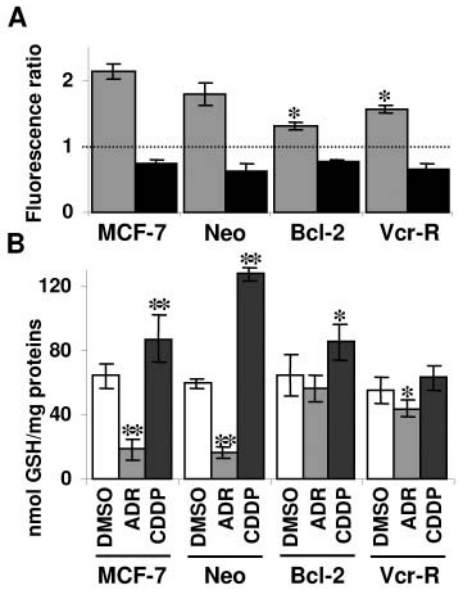

Figure 3. *Detection of ROS and GSH in MCF-7-derived cells during ADR and CDDP treatments. (A) ROS level detected by flow cytometry as DCF fluorescence ratio between treatment and DMSO (line) after 24 h-intoxication with 5 μM ADR (grey bars) or 50 μM CDDP (black bars). (B) Intracellular glutathione levels in the cell lines. Control was done with DMSO (open bars). Data are means ± SD (n=3), *p<0.05, **p<0.01 with respect to control.*

Bcl-2 proteins could be observed in the four cell lines after 24 h of intoxication with the two drugs (results not shown). PARP proteolysis, known as a late apoptosis marker, could not be detected at early treatment (24 h; data not shown) but appeared later (48 h) in MCF-7 and Neo cells only with ADR-treatment (Figure 2). A major PARP cleavage was detected in the presence of ADR or CDDP in Vcr-R cell line. No such cleavage could be detected either in ADR- or in CDDP-treated Bcl-2 cells.

Since high ROS level is known to induce cell death, we next evaluated oxidative stress in the four cell lines after 24 hours of treatment (Figure 3A). In agreement with its pro-oxidant activity, ADR induced an important generation of ROS in sensitive cells, whereas the increase was more moderate in the resistant ones, especially in Bcl-2 cells. These results support a previous report showing that Bcl-2 can act as an anti-oxidant protein when neurons are submitted to ADR treatment (16). Conversely, all cells were characterized by a low ROS level after 24 h of CDDP treatment, (Figure 3A).

Considering the major antioxidant function of GSH, the effect of the drugs on the glutathione status was evaluated. A 24-h ADR treatment triggered a 3-fold decrease in GSH level in MCF-7 and Neo cells, a slight decrease in Vcr-R cells, whereas in Bcl-2 cells the GSH level was not significantly affected (Figure 3B). Conversely, CDDP treatment significantly increased the GSH level in all cell lines except in Vcr-R cells.

Detection of early GSH efflux and ROS during ADR and CDDP treatments. Since no increase in GSSG could be detected during cell intoxication (data not shown), we have hypothesized that the observed loss of GSH could be achieved via GSH efflux from cells, which seems to be an early event of apoptosis (6, 17). As we observed programmed cell death within 24 h (Table I), GSH efflux was therefore determined within the frame of the first 12 h of intoxication (Figure 4). The results showed that ADR or CDDP provoked a GSH efflux in MCF-7 and Neo cells, which tended to diminish or stabilize after 8 h of treatment. Conversely, no GSH efflux was observed in Bcl-2 and Vcr-R cells, whatever the treatment. Furthermore, intracellular GSH remained essentially stable in all cells in the absence of the two drugs while ADR and CDDP treatments respectively reduced and increased its level in the two drug-sensitive cells. Intracellular GSH in Bcl-2 cells was not affected by ADR while CDDP treatment resulted in a 60% increase in this tripeptide. Neither ADR nor CDDP affected its level in Vcr-R cells.

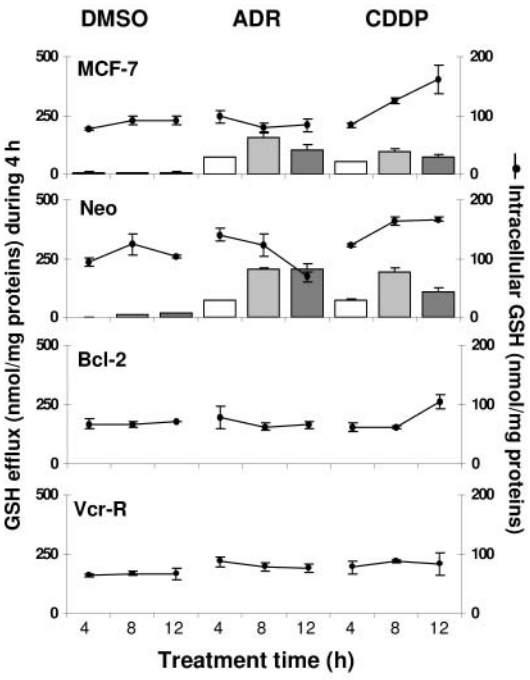

Figure 4. *GSH efflux and intracellular level in MCF-7-derived cells during ADR and CDDP treatments. Cells were incubated with ADR and CDDP for 4 h, 8 h, and 12 h, followed by a wash and a subsequent 4 h-incubation in Krebs-Henseleit buffer prior to glutathione efflux (bars) and intracellular GSH (●) quantification. Each bar represents the glutathione efflux measured in Krebs-Henseleit buffer after an initial cell incubation of 4 h (white bars), 8 h (pale grey bars) and 12 h (dark grey bars) with DMSO, ADR and CDDP. Data are expressed as means ± SD (n=3).*

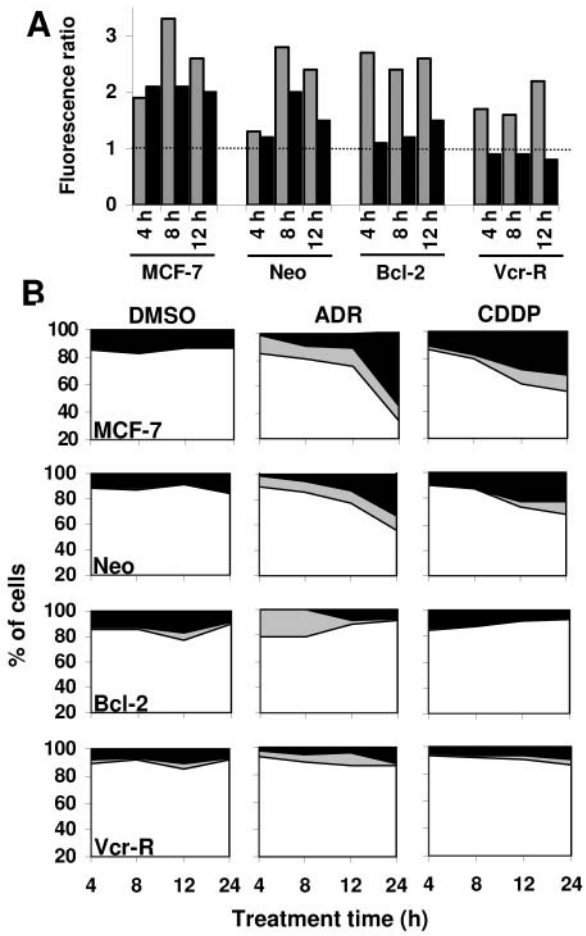

Figure 5. *ROS detection and cell death pattern in MCF-7-derived cells during ADR and CDDP treatments. (A) ROS level, expressed as DCF fluorescence ratio between treatment and control, was evaluated after 4, 8 and 12h-treatments of ADR (grey bars) and CDDP (black bars). (B) Percent of apoptosis (grey area), necrosis (black area) and living cells (white area) were determined by flow cytometry using Annexin-V assay, after 4, 8, 12 and 24 h-treatments.*

In order to evaluate the impact of GSH efflux on cell death induction, oxidative stress and apoptosis were measured within this time frame by flow cytometry. A rapid ROS induction with ADR treatment was detected in all cell lines (Figure 5A) and maintained for 24 h (Figure 3A). This induction was also detected with CDDP in sensitive cells, but to a lesser extend, was very modest and delayed in Bcl-2-transfected cells, and repressed in Vcr-R cells. A time-dependent induction of cell death was observed with the two treatments in MCF-7 and Neo cells, whereas no significant apoptosis or necrosis was detected in Bcl-2 and Vcr-R cells at any time (Figure 5B) consistent with the results obtained for 24 h-treatments (Table I).

Discussion

The purpose of this study was to evaluate the link between redox status and resistance to cell death induced by cytotoxic drugs in cancer cells. The relationship between the GSH status and resistance to ADR and CDDP in human carcinoma MCF-7-derived cells was characterized, especially by focusing on the contribution of Bcl-2 to the resistance to these drugs by using transfected MCF-7 cells.

Sensitive cells displayed a GSH depletion during ADR intoxication (Figure 3). This depletion cannot be attributed to conjugation to ADR, as previously reported (18). It could be due to consumption of GSH to counteract the oxidative stress induced by the rapid ROS formation that was detected. Additionally, the early GSH efflux observed could contribute to this reduction in GSH level. Compared to ADR, CDDP induced a lesser GSH efflux in early treatment in sensitive cells (Figure 4). A time-dependent increase in intracellular GSH was detected in the latter cells submitted to CDDP intoxication within 24 h. These results are in agreement with those of Chen *et al.* (19) who have observed an increase in GSH in MCF-7 cells treated with CDDP. Within an early time frame (up to 12 h of treatment), it was observed that CDDP increased the ROS

level in sensitive cells (Figure 5A). After 24 h of CDDP treatment, all cells were characterized by a low ROS level and high GSH content (Figure 3), and an adaptation process to the oxidative stress mediated by CDDP within these experimental conditions could therefore be postulated in agreement with Chen *et al.* (19).

Vcr-R is a MCF-7 subline overexpressing GST Pi and P-glycoprotein, which have been associated with multidrug resistance (20). This cell line, used here as a resistance model, is cross-resistant to ADR and CDDP. Treatments with these two drugs did not importantly affect the death pattern in Vcr-R cells (Figure 5B), which could be explained by a restrained ROS induction (Figures 3 and 5) associated with a relatively stable intracellular GSH (Figure 3). Unlike sensitive cells, no GSH efflux was observed in early treatments in Vcr-R (Figure 4). Taking into account that MRP1 mRNA have recently been detected in these cells (10), the results obtained here suggest that the resistance mechanism in Vcr-R may implicate multidrug resistance-associated proteins. MDR pathway may thus prevent oxidative stress, and consequently limit GSH level modulation. Despite this resistance, PARP cleavage was observed after 48 h in the presence of ADR and CDDP (Figure 2C) indicating that all pathways implicated in cell death induction were not entirely repressed in this cell type. This resistance type is independent of Bcl-2 overexpression (Figure 1) and may be essentially due to MRP function.

The Bcl-2 cell line was found to be the most resistant to ADR and CDDP (Figure 2A). The very low proportion of cell death during treatments in these cells supports the anti-apoptotic function of Bcl-2, which was similarly reported in the presence of anticancer drugs in a number of cell lines (21-23). The lack of PARP cleavage observed after 48 h in the presence of the two drugs in these cells is in accordance with the effect of Bcl-2 upstream the inhibition of caspases, which are responsible for PARP proteolysis. This observation underlines the differing mechanisms of resistance displayed by Vcr-R and Bcl-2 cells.

Furthermore, it has been reported that Bcl-2 overexpression increases GSH content (21, 24-26), but this observation seems to be cell type-dependent (27, 28) or oxidative stress-dependent (29). In the present experiments, a low ROS level was detected (Figure 3A), together with a steady-state level of GSH (Figure 3B) and an inhibition of GSH efflux (Figure 4) in ADR-treated Bcl-2 cells, which overall could contribute to cell survival. The inhibition of GSH efflux in Bcl-2 cells was also observed in mouse melanoma cells overexpressing Bcl-2 (7). The role of Bcl-2 in the inhibition of the release of free GSH and in the intracellular relocalization of the tripeptide to counteract cell death (30) will deserve further investigation to better understand its implication in drug resistance.

In summary, it is reported here that (i) Vcr-R cells resistance against the conventional anticancer drugs ADR and CDDP is independent of Bcl-2; (ii) Bcl-2 overexpression confers the strongest degree of resistance of MCF-7-derived cells; (iii) this resistance appears to be associated with GSH efflux inhibition, regardless of the cell lines used in the present study. The observations further highlight Bcl-2 as a prime pharmacological target to sensitize cancer cells to chemotherapeutic agents.

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