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Development of Resistance to Glutathione Depletion-Induced Cell Death in CC531 Colon Carcinoma Cells: Association with Increased Expression of Bcl-2

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ABSTRACT. The glutathione (GSH) level of CC531 rat colorectal cancer cells is readily decreased by exposure to buthionine sulfoximine (BSO), an inhibitor of GSH synthesis; at 25 μM BSO, these cells died in a non-apoptotic fashion. By continuous exposure of CC531 cells to increasing concentrations of BSO, we obtained a BSO-resistant cell line (CCBR25) that was 50 times more resistant to BSO than the parental cell line. Whereas the GSH content of CCBR25 and CC531 cells was similar, the former contained a much higher level of the Bcl-2 protein. After stable transfection of CC531 cells with the human *bcl-2* gene, the resulting Bcl-2-overexpressing cell line appeared to be 9 times more resistant to BSO than the parental cell line. These findings suggest that the Bcl-2 protein offers resistance against the cytotoxic effect of severe GSH depletion. BIOCHEM PHARMACOL **59**;12:1557–1562, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. glutathione; buthionine sulfoximine; Bcl-2; apoptosis; necrosis

The tripeptide GSH is an ubiquitous non-protein sulfhydryl important, for example, in the maintaining of intracellular redox balance and in detoxification of ROS§ and toxic xenobiotics [1, 2]. Lowering cellular levels of GSH, for instance by treatment with the GSH synthesis inhibitor BSO, enhanced the cytotoxic effect of various anticancer drugs [3, 4]. We recently reported that BSO pretreatment of a panel of human colorectal cancer cell lines *in vitro* increased the cytotoxicity of the alkylating compound melphalan 2- to 3-fold [5]. It has been assumed that the protective effect of GSH is due to GSH conjugation of melphalan [6]. However, we found no evidence for GSH conjugation of melphalan in several cell lines,‖ nor did we observe these conjugates in bile from patients whose livers were perfused with a relatively high dose of this agent [7]. Therefore, the protective effect of GSH is most likely not due to conjugation of melphalan. An alternative explanation is that a decrease in the intracellular level of GSH sensitizes cells to apoptosis-inducing stimuli [8–13]. In that case, it could be anticipated that severe loss of GSH by itself might lead to apoptosis; indeed, there have been several reports that fibroblasts and human neuronal cells died after GSH depletion [8, 14–16]. Anderson and colleagues [15] tested 18 human neuroblastoma cell lines and found that (apoptotic) cell death occurred once GSH levels decreased below 10% of the control level. Depletion of GSH resulted in increased formation of ROS, and cell killing was antagonized by antioxidants [15, 16]. In the hypothalamic neural cell line GT1-7, GSH depletion caused necrosis; transfection with the antiapoptotic proto-oncogene *bcl-2* resulted in strongly reduced sensitivity, ascribed to a decrease in the cellular generation of ROS [14].

We found that BSO also caused cell death in the rat colon carcinoma cell line CC531, which we have used in several *in vitro* and *in vivo* experiments [17, 18]. We selected a CC531 subline with high resistance to BSO-induced cell death; our study shows for the first time that GSH depletion can select for the clonal outgrowth of tumor cells with a marked increase in endogenous Bcl-2 expression.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The CC531 tumor cell line originates from an adenocarcinoma of the colon, syngeneic for WAG/Rij rats [19]. An established cell line was maintained in culture in RPMI-1640 medium (Dutch modification). The culture medium

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§ *Abbreviations:* BSO, buthionine sulfoximine; ROS, reactive oxygen species; PI, propidium iodide; and FITC, fluorescein isothiocyanate.

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was supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 μg/mL of streptomycin, and 50 IU/mL of penicillin (GIBCO, Life Technologies). The CC531 variants (CCBR25, CCNeo, and CCBcl-2) were cultured in the same tissue culture medium as the parental cell line. Stably transfected CC531 cells were maintained under 200 μg/mL of neomycin (G418, GIBCO), except during experimental procedures. The CCBR25 cell line was cultured in medium containing 25 μM BSO (Sigma).

GSH Analysis

Exponentially growing cells were washed with PBS, pH 7.4, and harvested by trypsinization. GSH was extracted by adding 5% (w/v) sulfosalicyclic acid to pelleted cells. GSH (including other eventual thiols) in the extract were assayed by the method of Ellman [20], with some previously reported modifications [21]. Total protein was determined by the method of Lowry *et al* [22]. The thiol content ("GSH content") was expressed as μmol per gram of protein.

Effect of BSO Exposure on GSH Content

To establish the effect of prolonged BSO treatment on intracellular GSH content, exponentially growing cells were treated for 24 hr with medium containing BSO. After the incubation period, cells were analyzed for GSH as described above.

Cytotoxicity Assessment

Cells were seeded at 250 cells/well (96-well plate) in 100 μL RPMI-1640 medium. After a 24-hr period, cells were treated for 24 hr with medium containing various concentrations of BSO (0.2–125 μM). Subsequently, the cells were washed twice and tissue culture medium was added at that time and again after 3 days. Cell viability was measured by the WST-1 assay (Roche) at four days after BSO treatment. WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) is an MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-like tetrazolium-based colorimetric assay used to measure cell survival and chemosensitivity. The IC_{50} indicates the concentration of BSO that led to a drop in WST-1 absorbance to 50% of the control value. The experiments were performed at least in triplicate.

Reflection Contrast Microscopy

CC531 cells were treated for 24 hr with 25 μM BSO and 24 hr later washed with PBS. They were fixed with 1.5% glutaraldehyde in cacodylate buffer pH 7.3 and embedded in Epon according to standard procedures [21]. Ultra-thin sections (60–80 nm) were prepared, stained with acridin orange, and visualized by reflection contrast microscopy (DM/RB microscope, Leica) [23].

Detection of Cell Death and ROS by Flow Cytometry

CC531 cells (25,000/well) were seeded in 6-well plates and BSO (25 μM final concentration) was added after a 24-hr attachment period. After an additional 24 hr, cells were washed twice and tissue culture medium was added. Cells were harvested by trypsinization 24 hr later and cell death was analyzed using the APOPTEST™–FITC kit (Nexins Research B.V), while ROS was measured as described below. Briefly, for the detection of cell death, cell samples were washed with PBS and resuspended in tissue culture medium containing 1.5 mM Ca^{2+} and, after a 1-hr acclimatization period, annexin V–FITC and PI were added (according to the manufacturer's specifications). After a 10-min incubation period, samples were measured on a FACScan (fluorescence-activated cell sorting) flow cytometer (Becton Dickinson), and three distinct populations were identified: vital (R1; annexin V–FITC-negative and PI-negative), apoptotic (R2; annexin V–FITC-positive and PI-negative), and necrotic (R3; annexin V–FITC-positive and PI-positive).

Hydroperoxide formation as indicator for the generation of ROS was measured by flow cytometry [24], using the non-fluorescent probe dihydrorodamine-123 (Molecular Probes), which is oxidized by ROS to the fluorescent metabolite rhodamine-123.

Transfection of the bcl-2 *Gene into CC531 Cells*

The full-length human *bcl-2* gene [25] was excised from the pBKKS vector and subcloned into the EcoR1 site of the pCMV (plasmid from cytomegalovirus) vector (provided by the Department of Medical Biochemistry, Sylvius Laboratories, Leiden, The Netherlands). Orientation of the *bcl-2* insert was verified by sequencing (Baseclear). CC531 cells were grown in 6-well tissue culture plates, and when approximately 50% confluency had been reached, cells were incubated for 24 hr with the liposomal transfection reagent FuGene (Roche) with pCMV-*neo* or pCMV-*bcl-2* according to the manufacturer's specifications. After the incubation period, the cells were washed and exposed to tissue culture medium containing 500 μg/mL of neomycin for two weeks. Neomycin-resistant clones were trypsinized and single cells were picked and grown out into single colonies.

Detection of Bcl-2 by Western Blotting

For detection of rat Bcl-2, we applied the rabbit polyclonal antibody (pAb), N-19 (Santa Cruz Biotechnology). The mouse monoclonal antibody (mAb) sc 7382 (Santa Cruz Biotechnology) and mAb clone 100 (Pharmingen) were raised against amino acids 1 to 205 and 41 to 54 of the human Bcl-2 protein, respectively, and used for the detection of Bcl-2 in the transfected cell line. Actin was detected by mAb clone C4 (Roche). The secondary antibodies rabbit anti-mouse and swine anti-rabbit were labeled with

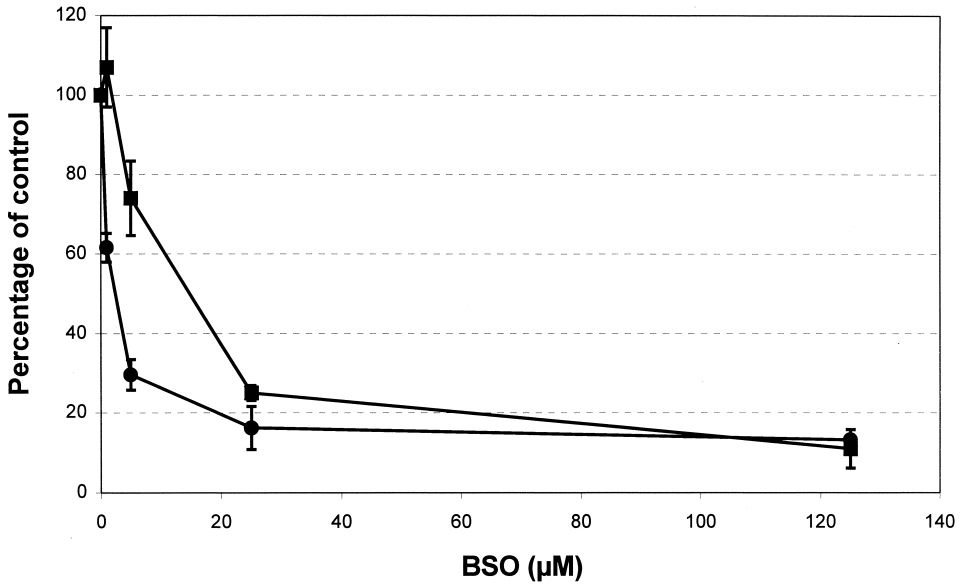

FIG. 1. Concentration-dependent decrease in GSH (●) and viability (■) of CC531 cells as a result of a 24-hr BSO exposure. Data points represent means ± SD (N = 3).

horseradish peroxidase (HRP) (DAKO). Subconfluent cell cultures were lysed in 1 M Tris pH 7.4, 50 mM EDTA, 500 mM NaCl, 1% (v/v) Nonidet P-40 supplemented with protease inhibitors PMSF (phenylmethylsulfonyl fluoride) and leupeptin (Sigma). The cell lysate was centrifuged at 10,000 *g* for 10 min at 4°. Detectable amounts of protein per lane were prepared and resolved by denaturating SDS–PAGE according to standard procedures. Following electrophoretic separation using 13% (v/v) SDS–PAGE, proteins were transferred to a nitrocellulose filter which was blocked for non-specific staining with 5% (w/v) non-fat dry milk for 60 min at 37°. Filters were incubated with primary antibody dissolved in PBS/5% (w/v) non-fat dry milk for 120 min at room temperature and then incubated with HRP-conjugated secondary antibody for 60 min at room temperature. Subsequently, the filters were incubated for 1 min with ECL (enhanced chemiluminescence) reagent and exposed to Hybond ECL film (Amersham Pharmacia Biotech).

RESULTS

Effect of GSH Depletion on Tumor Cell Viability

The GSH level of the CC531 colorectal cancer cell line, as measured with Ellmann's reagent, was substantially decreased as a result of a 24-hr exposure to BSO (Fig. 1). As GSH decreased, cytotoxicity was observed: already at 5 μM BSO (leading to a 70% drop in GSH at the end of the 24-hr incubation period), a 26% reduction in WST-1 absorbance (as compared to untreated cells) was detected 4 days after BSO treatment. Figure 2 shows images of ultra-thin sections from CC531 monolayers analyzed by reflection contrast microscopy, comparing untreated cells with a cell culture 24 hr after termination of BSO treatment. Although dead cells showed an altered nuclear texture, no signs of nuclear fragmentation were observed. Compared to vital cells, dead cells showed a rather irregular morphology and a strongly reduced cytoplasmic staining, but no notable reduction in

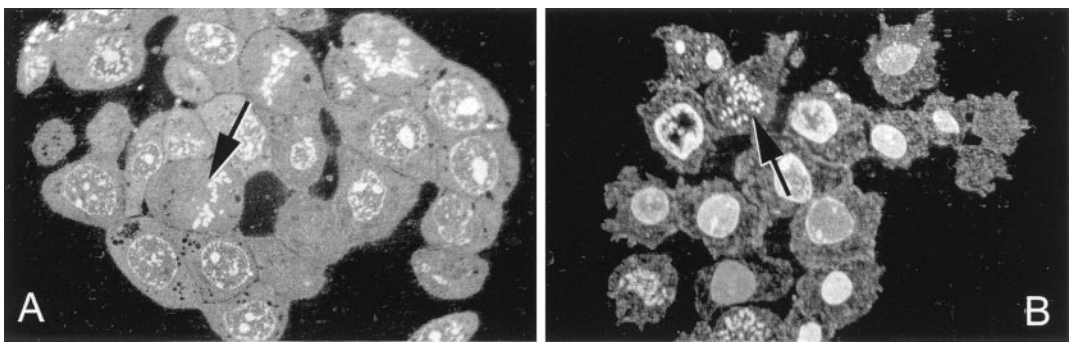

FIG. 2. Ultra-thin tissue sections of (A) vital and (B) non-apoptotic ("necrotic") CC531 cells 24 hr after termination of BSO treatment. The cells were treated for 24 hr with BSO (25 μM). After incubation, the cells were washed and tissue culture medium was added for an additional 24 hr. As shown in (B), the nucleus showed no sign of apoptotic nuclear fragmentation, and no cell swelling was observed. Arrows indicate mitotic chromosomes.

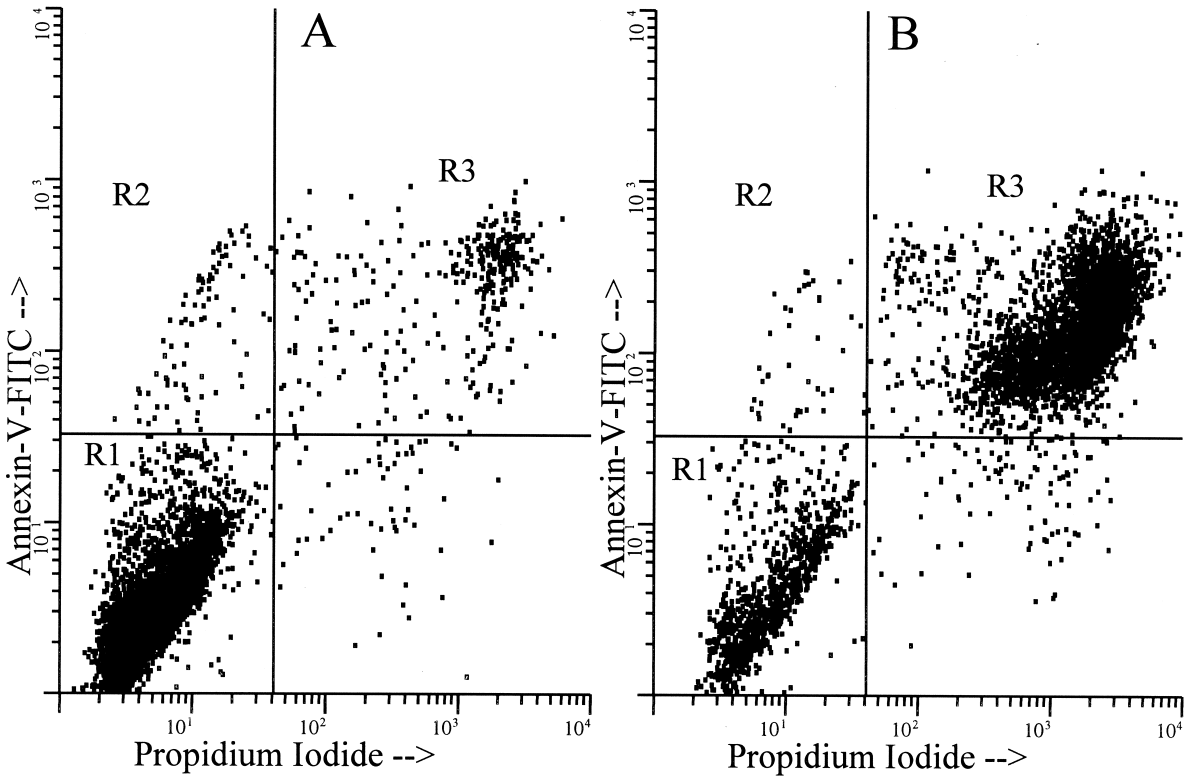

FIG. 3. Dot-plots of annexin V–FITC fluorescence versus propidium iodide (PI) fluorescence of both control (A) and BSO-treated (B) CC531 cells as assessed 24 hr after termination of BSO (25 μM) treatment. The dot-plot shows three distinct populations: viable (R1; annexin V–FITC-negative and PI-negative), apoptotic (R2; annexin V–FITC-positive and PI-negative), and necrotic cells (R3; annexin V–FITC-positive and PI-positive). After BSO treatment, the percentage of viable, apoptotic, and necrotic cells was 22%, 2%, and 76%, respectively. The percentage of viable, apoptotic, and necrotic cells in control samples was 90%, 2%, and 8%, respectively.

intercellular contacts. These data indicate, therefore, that BSO-treated cells died in a non-apoptotic fashion. Thus, the morphology of cell death, as evaluated by light microscopy, differed markedly from apoptotic CC531 cells as we observed after treatment with melphalan (data not shown).

Control and BSO-treated cell populations were also analyzed by flow cytometry after staining with annexin V–FITC (known to bind strongly to phosphatidylserine exposed on the surface of cellular membranes, notably those of apoptotic and necrotic cells) [5, 26] and propidium iodide (known to cross leaky cell membranes). As indicated by the very low number of cells in the annexin V–FITC-positive/PI-negative, i.e. apoptotic fraction (R2; 2%) and the massive uptake of PI (R3; 76%) 24 hr after BSO treatment (Fig. 3), GSH depletion-induced cell death clearly showed characteristics of necrosis.

Effect of Bcl-2 Overexpression on Resistance to BSO

We developed a BSO-resistant subline by continuous exposure of CC531 cells to, initially, 5 μM BSO, followed after two weeks by continuous exposure to 25 μM BSO. Treatment of the cells with increasing concentrations of BSO revealed that the selected subline (CCBR25) was 50-fold more resistant to BSO than the parental cell line (Table 1). In the absence of BSO, the GSH contents of CCBR25 and CC531 cells were similar (20.4 ± 9.7 and 20.4 ± 3.2 μmol/g protein, respectively). In the presence of BSO (25 μM), the GSH content of CCBR25 cells decreased to 4.6 ± 3.2 μmol/g protein (22.5% of the control CCBR25 level), very similar to that of the control cells (Fig. 1). Thus, the GSH level and the modulation of this level by BSO were similar in the CC531 and CCBR25 cell lines, indicating that resistance to BSO was not caused by changes in GSH metabolism, e.g. by reduced inhibition of γ-glutamyl cysteine synthetase (the rate-limiting enzyme in GSH synthesis). However, as shown in Fig. 4A, CCBR25

TABLE 1. Sensitivity of CC531 variants to BSO

Cell line	IC_{50} BSO (μM)	Resistance factor
CC531	16 ± 3	1
CCBR25	846 ± 489	50
CCNeo	20 ± 4	1
CCBcl-2	146 ± 61	9

Cells were exposed to various BSO concentrations for 24 hr. Subsequently, cells were washed and cell viability determined 4 days later by the WST-1 assay. Cytotoxicity (IC_{50}) is expressed as the concentration of BSO that led to a drop in WST-1 absorbance to 50% of control value. The resistance factor is calculated by dividing the IC_{50} BSO concentration of the CC531 variants by that of the parental CC531 cell line. Values are means ± SD. The experiments were performed at least in triplicate.

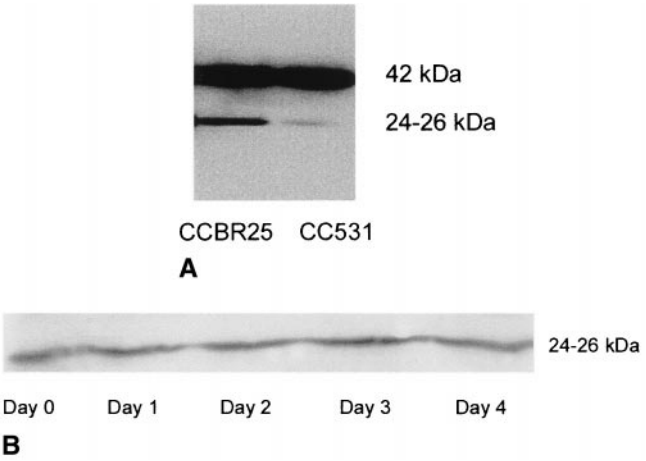

FIG. 4. Western blot analysis of the level of rat Bcl-2 (24–26 kDa) in the CC531 and CCBR25 cell lines. (A) A much higher expression of Bcl-2 was detected in the CCBR25 cell line compared to the CC531 cell line. Actin was detected at 42 kDa. (B) Expression of Bcl-2 in the CCBR25 cell line in the presence of BSO (25 μM) (day 0) and after BSO withdrawal for 1, 2, 3, and 4 days.

cells appeared to contain a much higher level of Bcl-2 protein. This was not due to a (reversible) effect of GSH depletion, because after prolonged BSO withdrawal we observed no decrease in the amount of Bcl-2 (Fig. 4B). Moreover, Bcl-2 was not up-regulated in the parental CC531 cells by a 24-hr treatment with BSO (25 μM) (as measured at the end of the BSO incubation; data not shown).

To confirm that Bcl-2 up-regulation protects against BSO-induced cell death, we stably transfected CC531 cells with a full-length human *bcl-2* gene. The selected human Bcl-2-overexpressing CC531 variant (CCBcl-2) (Fig. 5) appeared to be 9 times more resistant to BSO treatment (based on the IC_{50} value) than the parental cell line (Table 1). In the presence of 25 μM BSO, the GSH content of these CCBcl-2 cells decreased to 3.4 ± 0.4 μmol/g protein; this response is similar to that of both CC531 and CCBR25.

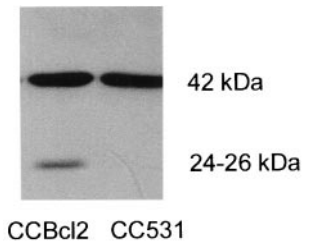

FIG. 5. Expression of human Bcl-2 (26 kDa) in CCBcl-2 and CC531 cells as measured by Western blot analysis. As shown, a high amount of the Bcl-2 protein was detected in the human Bcl-2-transfected CCBcl-2 cells, whereas no staining was detected in CC531 and CCNeo cells (not shown). Actin was detected at 42 kDa.

DISCUSSION

The present results show that GSH depletion of CC531 cells can select for the outgrowth of BSO-resistant tumor cells with an increased endogenous Bcl-2 expression. It was shown earlier that transfection of *bcl-2* may protect cells against cell death, including necrotic cell death [14, 27]. Kane and co-workers [14], using GT1-7 neural cells, reported that transfection of *bcl-2* prevented necrotic cell death induced by GSH depletion, presumably by decreasing the generation of ROS. Both in CC531 and in neuroblastoma cells [15], depletion of GSH resulted in increased formation of ROS. Preliminary experiments indicated that BSO treatment led to a 2.6-fold increase in intracellular ROS formation in the CC531 cells (data not shown), but this increase was similar in both CC531 and CCBR25, suggesting that Bcl-2 did not prevent ROS formation. Interestingly, Gardner and co-workers [28] clearly showed that although under their experimental conditions Bcl-2 could prevent ROS formation, this event appeared to be totally independent of the capacity of Bcl-2 to prevent apoptotic cell death. Therefore, our data do not exclude the possibility that Bcl-2 may act on pathways downstream of ROS formation.

The CCBR25 cells showed an approximately 5-fold higher resistance to BSO compared to CCBcl-2 cells overexpressing human Bcl-2 protein. Unfortunately, as CCBR25 express endogenous (rat) Bcl-2, we could not compare the relative amounts of Bcl-2 in these two cell lines, because rat and human Bcl-2 require different antibodies for their (quantitative) detection. Moreover, rat and human Bcl-2 may differ in various subtle ways, possibly leading to differences in their capacity to counteract BSO treatment.

In conclusion, we have isolated a CC531 variant that is resistant to cell death induced by GSH depletion; endogenous Bcl-2 has been shown to be a selective factor in BSO resistance.

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