

Possible Role of Inhibition of Glutathione *S*-Transferase in the Partial Reversal of Chlorambucil Resistance by Indomethacin in a Chinese Hamster Ovary Cell Line¹

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ABSTRACT

We have reported previously the isolation and characterization of a Chinese hamster ovary cell line, designated CHO-Chl', which exhibits resistance to bifunctional nitrogen mustards while maintaining sensitivity to a range of other alkylating agents and chemotherapeutic drugs. This enhanced drug resistance is associated with a greater than 40-fold increase in the level of expression of an alpha class (YcYc) glutathione *S*-transferase (GST) as compared to the parental, CHO-K1, cell line. Here, we have purified GST from CHO-Chl' cells and show that the nonsteroidal antiinflammatory drug indomethacin acts as an inhibitor of enzyme activity. Indomethacin at 500 μ M causes no significant decrease in colony forming ability of either CHO-K1 or CHO-Chl' cells. However, the cytotoxicity of chlorambucil is potentiated 5.5-fold in CHO-Chl' cells, but only 2.5-fold in CHO-K1 cells following preexposure to 500 μ M indomethacin. In contrast, the antiinflammatory agent acetylsalicylic acid failed to inhibit the activity of purified GST and caused no potentiation of chlorambucil toxicity, suggesting that the potentiation by indomethacin is not due to the effects of this drug on prostaglandin synthesis. These studies provide further evidence that GSTs may be involved in the development of resistance to bifunctional alkylating agents and suggest that indomethacin, or agents with similar activities, may be of value as an adjunct to chemotherapy in some patients with tumors resistant to treatment with alkylating agents.

INTRODUCTION

Bifunctional alkylating agents form an important part of many regimens used to treat patients with a range of solid tumors and hematological malignancies. Often initial treatment produces a marked decrease in the tumor load but this is followed, after a variable period, by the emergence of tumor cells which are more resistant to subsequent treatment with the same class of drugs. Studies using both rodent and human cell lines have suggested that a variety of cellular mechanisms underlie the development of resistance to bifunctional alkylating agents. These include a decrease in drug uptake (1), an increase in drug detoxification (particularly involving glutathione conjugation) (2), and enhanced DNA repair (3).

Several studies which have characterized cell lines resistant to alkylating agents have demonstrated enhanced expression of a group of cytosolic enzymes identified as the GSTs³ (4-6). It has been suggested that these enzymes may be directly involved in drug detoxification by catalyzing conjugation of drugs to glutathione. This hypothesis has been supported by experiments which have demonstrated the formation of glutathione-mel-

phalan conjugates in crude liver extracts containing GSTs (7), although this has not yet been demonstrated using purified enzyme preparations.

We have reported previously the isolation of a Chinese hamster cell line (designated CHO-Chl') which displays an approximately 20-fold increase in resistance to chlorambucil as compared to the parental form (CHO-K1) (8). Resistance to melphalan and mechlorethamine is also increased, but sensitivity to other classes of alkylating agents, such as bis(2-chloroethyl)-1-nitrosourea, ethyl methanesulfonate, cyclophosphamide, and mitomycin C, is not affected. CHO-Chl' cells have been shown to express a 3-fold increase in activity of cytosolic GST against the model substrate CDNB as compared to CHO-K1 cells (9). Immunoblotting and immunocytochemical studies have shown that this increase is associated with a marked overexpression of a *M*_r 28,000 protein which reacts with antiserum raised against the basic form of human GST. This protein has been purified and shown to be an alpha class GST comprising YcYc subunits (16).

Although an association between enhanced resistance to alkylating agents and elevated GST activity has been reported by several groups, few studies have demonstrated any reversal of resistance caused by inhibiting GST activity in intact cells. Studies on GST purified from human platelets have shown that the nonsteroidal anti-inflammatory agent, indomethacin, acts as an inhibitor of GST activity (10). Similar experiments have shown inhibition of GST purified from rat liver using the same drug (11). Indomethacin is an extremely well tolerated drug with few serious side effects and no known cytotoxic actions when used in clinical practice.

Here, we describe the effect on chlorambucil toxicity of pretreating CHO-K1 and CHO-Chl' cells with indomethacin. We show that a substantial reversal of the drug resistant phenotype of CHO-Chl' cells can be achieved.

MATERIALS AND METHODS

Cell Culture. Cells were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 5% newborn calf serum, glutamine (3 mM), and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μ g/ml; and nystatin, 50 units/ml). Cells were grown as monolayers at 37°C in a humidified atmosphere containing 5% CO₂.

Toxicity of Indomethacin and Acetylsalicylic Acid. Clonogenic assays were performed to assess the toxicity of indomethacin against CHO-K1 and CHO-Chl' cells. Cells were removed with Dulbecco's phosphate buffered saline (without calcium and magnesium, pH 7.4) containing 25 mM EDTA, plated in 35-mm Petri dishes, and incubated for 4 h at 37°C to allow cell adhesion. Indomethacin was added to give a final concentration ranging from 0 to 1 mM. After 2 h the indomethacin solution was replaced with fresh medium and the cells were incubated for 12 days. Medium was aspirated, and the cells were fixed with 20% acetic acid in methanol and stained with crystal violet (0.4% in water). Colonies over 1 mm in diameter were counted and the results were expressed as a percentage of the colonies present in a control exposed to medium alone. Similar experiments were performed to assess the

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³ GSTs, glutathione *S*-transferases; CHO, Chinese hamster ovary; CDNB, 1-chloro-2,4-dinitrobenzene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS/Tween; Tris buffered saline containing 0.5% Tween; D₃₇, the dose of chlorambucil required to reduce survival to 37% of control values.

toxicity of acetylsalicylic acid up to a concentration of 1 mM.

Effect of Indomethacin and Acetylsalicylic Acid on Chlorambucil Toxicity. Clonogenic assays were performed using a technique similar to that described above. Indomethacin was added to a final concentration of 500 μM . After 1 h, various concentrations of chlorambucil were added (without replenishing the medium) and the incubation was continued for 1 h further. The medium was replaced and the cells were incubated until colonies developed. The D_{37} was calculated for each cell line, with and without indomethacin. Similar experiments were performed using acetylsalicylic acid.

Immunoblotting of Crude Lysates. CHO-K1 and CHO-Chl^r cells were harvested as above and washed twice in Dulbecco's phosphate buffered saline (without calcium and magnesium, pH 7.4). Cells were lysed by sonication in 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol. SDS-PAGE was performed according to the method of Laemmli (12). Proteins were transferred to nitrocellulose using the method of Towbin *et al.* (13). The nitrocellulose was washed three times in TBS/Tween. After 1 h, antiserum raised against the human forms of GST was added to the buffer to give a final dilution of 1:1000. Incubation was continued at room temperature for 4 h with gentle agitation. The filter was washed three times in TBS/Tween and incubated for 4 h with 5 μCi ¹²⁵I-labeled protein A. After extensive washing with TBS/Tween the filter was dried before autoradiography for 16 h at -70°C with intensifying screens.

Purification of GST from CHO-Chl^r Cells. CHO-Chl^r cells (7×10^8) were lysed in 10 mM Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol (buffer A), using a glass/Teflon homogenizer (ten strokes). The homogenate was centrifuged at 100,000 $\times g$ for 1 h at 4°C. The supernatant was passed at a flow rate of 5 ml/h down a 6.4-ml *S*-hexylglutathione affinity column prepared according to the method of Mannervik (14). The column was washed with 20 bed volumes of buffer A containing 200 mM NaCl (buffer B). GST was eluted using a linear 30-ml gradient of 0-1 mM *S*-hexylglutathione in buffer B. Fractions were collected and tested for GST activity using the method of Habig and Jakoby (15), adapted for use on a multichannel plate reading spectrophotometer. The purity and composition of the eluted fractions were assessed by SDS-PAGE.

Effect of Indomethacin and Acetylsalicylic Acid on GST Activity. Purified GST was diluted in 10 mM potassium phosphate buffer, pH 6.5, to give an activity of approximately 80 units/ml when assayed according to the method of Habig and Jakoby. (One unit is defined as the amount of enzyme required to conjugate 1 nmol of CDNB/min using 1 mM concentrations of CDNB and glutathione at 25°C.) Diluted enzyme (450 μl) was mixed with 50 μl of indomethacin dissolved in ethanol and the mixture was incubated at 25°C for 10 min. A reagent mixture (500 μl), containing 2 mM glutathione and 0.8 mM CDNB in 10 mM potassium phosphate buffer, pH 6.5, was added to the incubation mixture and the rate of change in absorbance at 340 nm was measured using a Shimadzu UV-160 spectrophotometer. Triplicate assays were performed for concentrations of indomethacin in the incubation mixture of 0.167, 0.334, 0.5, and 0.667 mM. Control assays were performed in which ethanol alone was incubated with the diluted enzyme. Similar experiments were performed using acetylsalicylic acid to give a final concentration in the incubation mixture of 1 mM.

RESULTS

Toxicity of Indomethacin and Acetylsalicylic Acid. Exposure of CHO-K1 or CHO-Chl^r cells to concentrations of indomethacin of up to 500 μM produced minimal toxicity (Fig. 1). Acetylsalicylic acid produced similar results (data not shown).

Effects of Indomethacin and Acetylsalicylic Acid on Chlorambucil Toxicity. Exposure of CHO-K1 cells to 500 μM indomethacin for 1 h prior to treatment with chlorambucil reduced the D_{37} from 20 to 8 $\mu\text{g}/\text{ml}$. The same treatment of CHO-Chl^r cells reduced the D_{37} from 100 to 20 $\mu\text{g}/\text{ml}$ (Fig. 2).

Immunoblotting of Crude Lysates. Immunoblotting using antisera against the human basic forms of GST, showed a greater than 40-fold increase in the level of the Yc subunit in

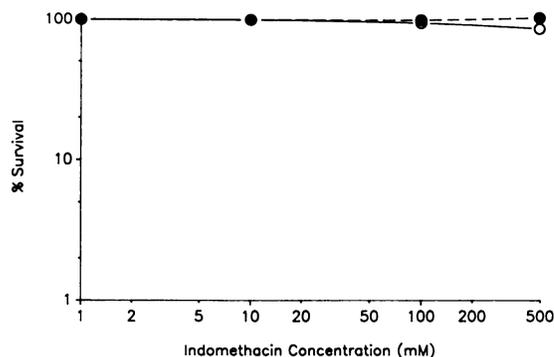


Fig. 1. Effect of indomethacin on survival of CHO-K1 (●) and CHO-Chl^r (○) cells. Points represent the mean of three independent experiments. The standard error for each point was less than 6%.

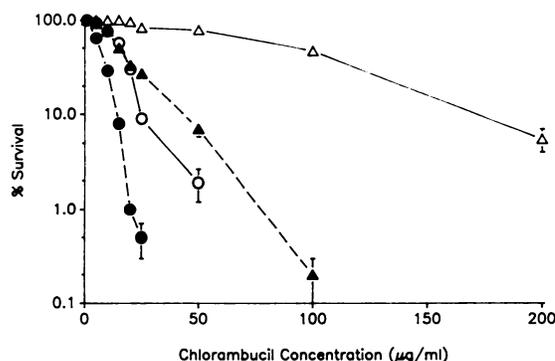


Fig. 2. Effect of indomethacin preincubation on survival of CHO-K1 and CHO-Chl^r cells. \blacktriangle , CHO-K1 with no preincubation; \bullet , CHO-K1 after preincubation; \triangle , CHO-Chl^r with no preincubation; \circ , CHO-Chl^r after preincubation. Points represent the mean \pm SE (bars) of three independent experiments.

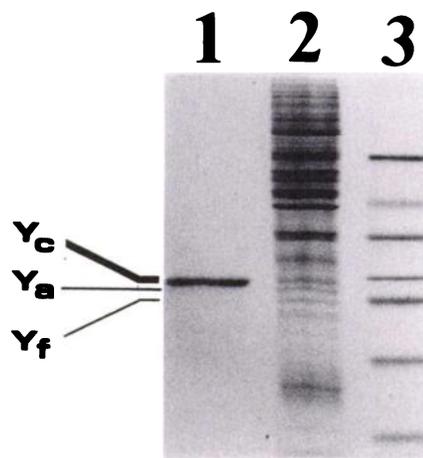


Fig. 3. SDS-PAGE of crude cytosol from CHO-Chl^r cells (Lane 2) and GST purified by *S*-hexylglutathione affinity chromatography (Lane 1). Positions of Yc (thick bar) and Ya and Yf subunits (thin bars) are indicated. Lane 3 contains molecular weight standards.

the CHO-Chl^r cells compared to CHO-K1 cells, and a lesser increase in the level of the Ya subunit (Fig. 4).

Purification of GST from CHO-Chl^r Cells. Purification by affinity chromatography produced a mixture of three proteins with relative molecular weights of approximately 28,000, 27,000, and 22,000 (Fig. 3). The predominant form, with a molecular weight of 28,000, has been shown in a previous study to be the hamster homologue of the rat Yc subunit (16). The molecular weights of the M_r 27,000 and M_r 22,000 bands indicate that they represent the basic Ya, and acidic Yf subunits, respectively.

1 2



Fig. 4. Immunoblot of crude cytosol from CHO-K1 (Lane 2) and CHO-Chl^r cells (Lane 1) using antiserum raised against human basic forms of GST. The upper band in Lane 1 represents Yc subunits. The lower band represents Ya subunits.

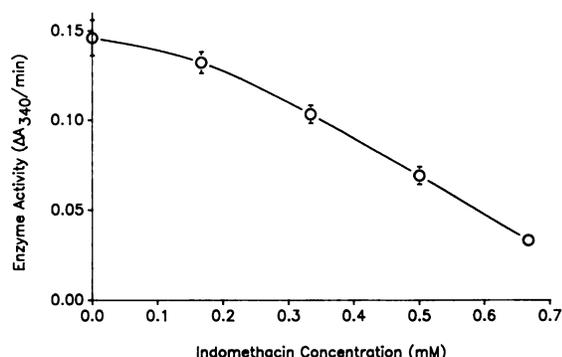


Fig. 5. Indomethacin inhibition of GST purified from CHO-Chl^r cells. Points represent the mean \pm SE (bars) of three independent experiments.

Effect of Indomethacin and Acetylsalicylic Acid on GST Activity. Using the model substrate CDNB at a concentration of 0.4 mM, inhibition of GST activity was assayed for a range of indomethacin concentrations. From a plot of enzyme activity against inhibitor concentration, the concentration of indomethacin required to produce 50% inhibition of activity was calculated to be 450 μ M (Fig. 5).

Acetylsalicylic acid produced no effect on GST activity up to a concentration of 1 mM.

DISCUSSION

The cytosolic GSTs are dimeric proteins which fall into three distinct groups classified according to their isoelectric points as basic, near neutral, and acidic (also termed alpha, mu, and pi, respectively). Within each group, subunits have been identified which can form either hetero- or homodimers. For example, Ya and Yc subunits may combine to form enzymes within the basic group (17).

In earlier studies aimed at characterizing the mode of resistance in the CHO-Chl^r subline, we reported that resistance was associated with the enhanced expression of cytosolic GST. More recent observations confirm that the 3-fold increase in bulk transferase activity (assessed by using CDNB as a model substrate) is mainly due to increased expression of a basic form of the enzyme which cross-reacts with antiserum raised against the rat Yc subunit (16). There are relatively minor increases in

the expression of an acidic transferase which cross-reacts with antiserum raised against human pi transferase, and of a basic form which cross reacts with Ya antiserum.

Several other studies have reported a similar correlation between resistance to alkylating agents and an elevation in levels of cytosolic GST activity, both in cell lines (4–6) and in tumor biopsies (18). The most obvious explanation for this association is that these enzymes are directly involved in catalyzing the formation of adducts between the alkylating agent and glutathione. The existence of such adducts has been demonstrated for melphalan in studies using cytosol containing GSTs (7), but the direct involvement of a specific GST in this reaction has not been proved. In an attempt to provide evidence for the role of GSTs in this form of drug resistance, we decided to study the effect of inhibiting GST activity on the sensitivity to chlorambucil of the CHO-Chl^r subline. We chose to use indomethacin as the inhibitor of enzyme activity because it is a drug which is well established in clinical practice and is associated with few toxic side effects. Acetylsalicylic acid was chosen as a negative control because it is a potent nonsteroidal anti-inflammatory drug but does not inhibit GST activity (10).

GST was purified from CHO-Chl^r using affinity chromatography. The *S*-hexylglutathione-agarose affinity matrix used has been shown in previous studies to be specific for GSTs (19, 20) and to yield samples of high purity, especially if low concentrations of *S*-hexylglutathione are used to elute the enzyme from the column. Resolution of the eluted proteins by SDS-PAGE revealed the presence of one major and two minor bands. Previous studies have shown that the major band represents the Yc subunit.

Indomethacin was shown to inhibit the activity of the purified GST. The degree of inhibition was approximately proportional to the concentration of indomethacin present. A concentration of 450 μ M was found to give a 50% reduction in activity at a CDNB level of 0.4 mM. Acetylsalicylic acid was shown to have no effect on GST activity up to a concentration of 1 mM.

Clonogenic assays revealed a 5.5-fold difference in sensitivity between CHO-Chl^r and CHO-K1 cells, using a 1-h exposure to chlorambucil. This is a somewhat lower level of relative resistance than is seen with 24 h of exposure. Indomethacin at concentrations of up to 500 μ M had little cytotoxic effect on either CHO-K1 or CHO-Chl^r cells in the absence of chlorambucil. However, preincubation of either cell type with 500 μ M indomethacin produced a significant increase in the cytotoxic effect of chlorambucil. In a separate series of experiments, this potentiation of chlorambucil toxicity was shown to be dependent on the dose of indomethacin used (data not shown). Preincubation with acetylsalicylic acid produced no significant change in the D₃₇ for chlorambucil in either CHO-K1 or CHO-Chl^r cells.

The observation that an inhibitor of GST activity is capable of causing a more marked increase in the cytotoxic action of chlorambucil against CHO-Chl^r cells than against CHO-K1 cells provides further evidence that the enhanced expression of cytosolic GSTs may be a significant factor in the development of resistance to alkylating agents in this subline. The smaller degree of potentiation in CHO-K1 cells possibly reflects the presence of a lower level of cytosolic GST in this line. These observations do not, however, exclude the possibility that indomethacin is acting through an unrelated mechanism. Inhibition of cyclooxygenase is unlikely to be responsible in view of the lack of potentiation seen using acetylsalicylic acid, another cyclooxygenase inhibitor. Experiments with the nonsteroidal anti-inflammatory drug, zomepirac, a structural analogue of

indomethacin, show intermediate effects on both GST inhibition and chlorambucil potentiation (data not shown). This lends further support to the theory that specific interaction with GST underlies potentiation of chlorambucil toxicity.

The finding that indomethacin is capable of causing partial reversal of resistance in a chlorambucil resistant cell line suggests that this drug may possibly be of value as an adjunct to chemotherapy in patients with tumors resistant to alkylating agents. If this is the case, the effect may be limited to those patients in which resistance is associated with an elevation of cytosolic GST. The differential potentiation of toxicity in cells expressing different levels of GST suggests that there may be a "therapeutic index" for this form of treatment augmentation. Studies are currently under way to investigate this hypothesis.

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