Pergamon

European Journal of Cancer Vol. 33, No. 3, pp. 447–452, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(96)00434-0

Original Paper

Role of O^6-methylguanine-DNA Methyltransferase, Glutathione Transferase M3-3 and Glutathione in Resistance to Carmustine in a Human Non-small Cell Lung Cancer Cell Line

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O^6-methylguanine-DNA methyltransferase (MGMT), glutathione transferase (GST) M3-3 and glutathione (GSH) have all been implicated in the resistance of cells to the cytostatic drug carmustine. U1810, a human non-small cell lung cancer cell line, expresses all of these putative resistance factors. The U1810 cells show a 4.4-fold lower sensitivity to carmustine compared with the U1690 cell line, a human small cell lung cancer cell line lacking detectable levels of both MGMT and GST M3-3. We investigated the effect of the MGMT inhibitor O^6-benzylguanine, the GST inhibitor ethacrynic acid and the GSH synthesis inhibitor D,L-buthionine-*S,R*-sulfoximine (BSO) on the cytotoxicity of carmustine to U1810 cells. No potentiation to carmustine was observed after treatment with ethacrynic acid, while a 2-fold potentiation was found after exposure to O^6-benzylguanine. Depletion of GSH with BSO showed a similar sensitising effect as that obtained with O^6-benzylguanine. Thus, MGMT and GSH are the predominant resistance factors to carmustine in the U1810 cell line, whereas it is unclear whether GST M3-3 plays any role.

Key words: lung cancer, drug resistance, carmustine, O^6-methylguanine-DNA methyltransferase, glutathione transferase, glutathione

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INTRODUCTION

THE DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), appears to contribute to chloroethylnitrosourea (CNU) resistance in tumour cells. The MGMT protein removes O^6-chloroethylguanine monoadducts by transfer of the alkyl group to a cysteine residue in the active centre of the protein itself, and thereby prevents the formation of DNA cross-links [1]. Cells lacking this protein are more sensitive to CNUs than those with high levels of the protein [1]. Tumour cells with high MGMT levels can be sensitised to CNUs by inactivation of MGMT using the inhibitor O^6-benzylguanine [2]. However, in some tumours, no correlation has been found between expression of MGMT and resistance to nitrosoureas [3, 4]. This suggests that other resistance mechanisms also contribute to CNU resistance.

The glutathione transferases (GSTs) are a family of cytosolic isoenzymes catalysing the conjugation of a variety of electrophilic compounds with glutathione (GSH). They have been divided into four classes according to their primary structure: alpha, mu, pi and theta [5, 6]. Several investigations have shown that GST-mediated conjugation of alkylating agents to GSH contributes to drug resistance in tumour cells [7]. Treatment with the GST inhibitor ethacrynic acid, a diuretic drug, has been shown to increase toxicity of alkylating agents in tumour cells [8–10]. Expression of the class mu isoenzyme GST M3-3 has been found to be correlated with resistance to carmustine. Smith and associates [10] found that a rat glioma subline, which was resistant to carmustine, had lower total GST activity but higher

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Received 9 Feb. 1996; revised 9 Aug. 1996; accepted 19 Aug. 1996.

levels of the class mu transferases compared to sensitive cells. We have shown in an earlier study that carmustine is inactivated by a denitrosation reaction catalysed by human GST M3-3 but not by GST M1-1 [11].

Increased levels of GSH may also contribute to drug resistance in tumour cells. GSH is the main cellular non-protein thiol, which detoxifies electrophilic substances by GST-catalysed or spontaneous conjugation. GSH can also quench the cross-linking reaction of the CNU induced monoadduct *in vitro* in the absence of GST [12]. The cellular levels of GSH can be reduced by several chemicals. Since ethacrynic acid is a substrate for GST-mediated GSH conjugation, it also lowers the GSH levels [8]. D,L-Buthionine-*S,R*-sulfoximine (BSO) acts as a direct inhibitor of the synthesis of GSH, by blocking γ-glutamyl cysteine synthetase, the rate-limiting enzyme in GSH biosynthesis [13].

The aim of this study was to investigate the significance of expression of MGMT, GST M3-3 and GSH on cellular sensitivity to carmustine. For this purpose, we chose the non-small cell lung cancer cell line U1810, which expresses both MGMT [14] and GST M3-3 [11]. The U1810 cells are more resistant to carmustine than a small cell lung cancer cell line (U1690) which lacks detectable levels of both MGMT and GST M3-3 [11, 14]. By inactivating MGMT and GST or by depleting GSH, we aimed to determine whether one or more of these mechanisms are important for resistance to carmustine in the U1810 cells. Through manipulation of these factors in different combinations, it was possible to elucidate how these putative resistance factors interact.

MATERIALS AND METHODS

Drugs and chemicals

Carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; (BCNU)] was obtained from Bristol-Myers Squibb Laboratories, Syracuse, New York, U.S.A. Immediately before drug incubations, 3 mg of carmustine was dissolved in 40 μl 99.5% alcohol and diluted in cell culture medium supplemented with 10% fetal calf serum (FCS) to the desired drug concentrations. Ethacrynic acid, obtained from Merck, Rahway, New Jersey, U.S.A., was dissolved and diluted in cell culture medium. BSO was obtained as a powder from Chemalog Chemical Dynamics Corp., South Plainfield, New Jersey, U.S.A. O^6-benzylguanine, kindly provided by Dr R. Moschel, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, Maryland U.S.A., was dissolved in DMSO and diluted in cell culture medium.

Cell culture

The U1810 cell line derived from a non-small cell lung cancer and the U1690 cell line derived from a small cell lung cancer were kindly provided by Dr Jonas Bergh, Department of Oncology, Uppsala University, Sweden [15]. The cells were grown as monolayer cultures in Eagle's Minimal Essential Medium (MEM) with Earle's salts (Flow Laboratories, Rickmansworth, U.K.) supplemented with 10% FCS and 2 mM L-glutamine. Benzylpenicillin (125 IU/ml) and streptomycin (125 μg/ml) were added to the cell culture media.

Determination of GSH content

The U1810 cells were treated with either BSO, O^6-benzylguanine or both in combination, as described below. Cells in monolayer culture were first washed with saline containing 2 mM EDTA. The cells were precipitated with 5% trichloroacetic acid in 12.5 mM EDTA on ice for at least 30 min. The content of GSH in the cells was determined by the method of Tietze [16] and related to the protein content of each cell sample.

Drug treatments and cytotoxicity

Drug-induced cytotoxicity was measured as inhibition of colony formation. Appropriate numbers (100–6400 cells/dish) of U1810 and U1690 cells were plated in 6 cm Petri dishes overnight to attach. The cells were either treated with carmustine immediately or pre-incubated with one or more of the following drugs: 10 μM BSO for 24 h, 5 μM O^6-benzylguanine for 2 h or 10 μM ethacrynic acid for 15 min, and then incubated with various concentrations of carmustine for 2 h in complete medium. When cells were exposed to ethacrynic acid, this drug was also present during the carmustine treatment. In some experiments, cells were exposed to 5 μM O^6-benzylguanine for 20 h following treatment with carmustine. After carmustine treatment, the cells were grown in fresh drug-free medium with 10% FCS and 2 mM L-glutamine for 14 days. The dishes were rinsed with phosphate-buffered saline (PBS) (pH 7.4), fixed with ethanol and stained with Giemsa solution, and the surviving fraction was calculated as the ratio of plating efficiency in dishes containing drug-treated cells over the plating efficiency in dishes with untreated cells. Cytotoxicity is expressed as the drug concentration (IC_{50}) inhibiting 50% of the colony formation of the cells.

RESULTS

Effect of O^6-benzylguanine on carmustine sensitivity

In order to examine the role of MGMT in U1810 cells' resistance to carmustine, we have used O^6-benzylguanine which is highly effective in depleting MGMT activity [2]. Treatment of U1810 cells with a non-toxic dose of 5 μM O^6-benzylguanine for 2 h reduced the MGMT activity to 1% of the control value (specific activity was 0.58 pmol/mg protein in untreated cells), and made the cells nearly twice as sensitive to carmustine calculated on the basis of the IC_{50} values (Figure 1). As expected, pretreatment with O^6-benzylguanine of the U1690 cell line, lacking expression of MGMT, showed no effect (Figure 2a). The U1690 cells showed a 4.4-fold higher sensitivity to carmustine than the U1810 cells (Figure 2a), which is in agreement with previous results [14]. U1810 cells were also exposed to O^6-benzylguanine both 2 h before and for 20 h after removal of carmustine, to maintain the MGMT depletion during the time required for establishment of DNA cross-links [17]. This treatment made the cells three times more sensitive to carmustine (Figure 2a).

Effect of ethacrynic acid on carmustine sensitivity

The inhibitory effect of ethacrynic acid on GST activity has previously been investigated, and it has been found that class mu GST M1-1 is the most sensitive enzyme with an I_{50} value (I_{50} is the concentration giving 50% inhibition of the enzyme) of 1 μM, while the alpha and pi class enzymes GST A1-1 and GST P1-1 have I_{50} values of 10 and 15 μM,

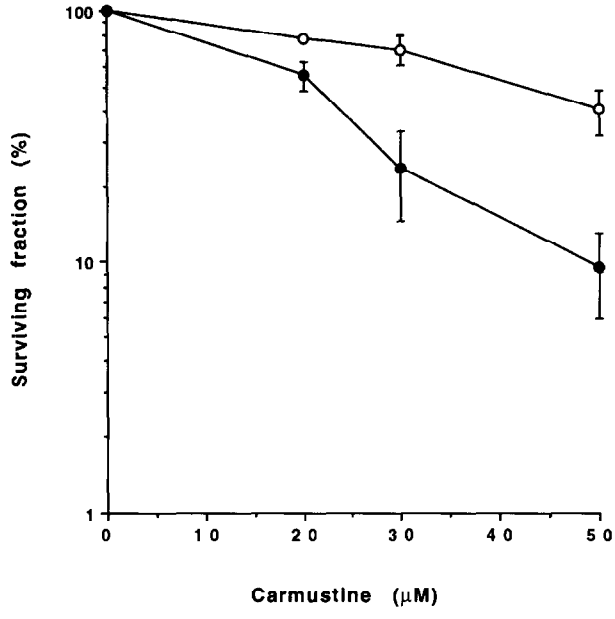

Figure 1. Effect of O^6-benzylguanine on carmustine toxicity. U1810 cells were pre-incubated for 2 h with 5 μM O^6-benzylguanine and then incubated with various concentrations of carmustine. No O^6-benzylguanine (○); 5 μM O^6-benzylguanine (●). Mean values of four separate experiments, bars indicate SEM.

respectively [9]. More recently, GST M3-3 has been reported to have an I_{50} value of 5 μM [18]. In the present study, we pre-incubated the cells with 10 μM ethacrynic acid for 15 min, a concentration which lacked toxic effects, before treatment with carmustine for 2 h in the presence of the same concentration of ethacrynic acid. No potentiation of carmustine cytotoxicity by ethacrynic acid was observed; when the IC_{50} values were compared a dose modulating factor of 1.03 was obtained. Adding ethacrynic acid to cells during the last 15 min of the 2 h treatment with O^6-benzylguanine and during the incubation with carmustine did not further increase the sensitivity of U1810 cells to carmustine (data not shown). Preliminary results show no inhibitory effect of 5 μM O^6-benzylguanine on GST M3-3 (data not shown).

Effect of BSO on sensitivity to carmustine

The U1810 cells were found to have a GSH concentration of 102.2 ± 27.0 nmol/mg protein (mean ± S.D.), while the U1690 cells have a GSH concentration of 59.4 ± 9.4. Increased GSH concentration may thus contribute to the relative resistance to carmustine of U1810 cells. Treatment of U1810 cells with 10 μM BSO for 24 h depleted the GSH level to 13% of the control level, while O^6-benzylguanine treatment had no effect on the GSH level. Treatment of the cells with both BSO and carmustine further enhanced the depletion of cellular GSH by 40%. Incubation with BSO decreased the plating efficiency to a mean of 50% compared to medium alone, and in order to compensate for this effect, the relative plating efficiency of control cells treated with BSO only was normalised to 100%. The carmustine sensitising effect of BSO on human glioma cells has been shown to be independent of the cytotoxic effects of treatment with BSO alone [19]. A 24-h BSO pretreatment sensitised the U1810 cells approximately

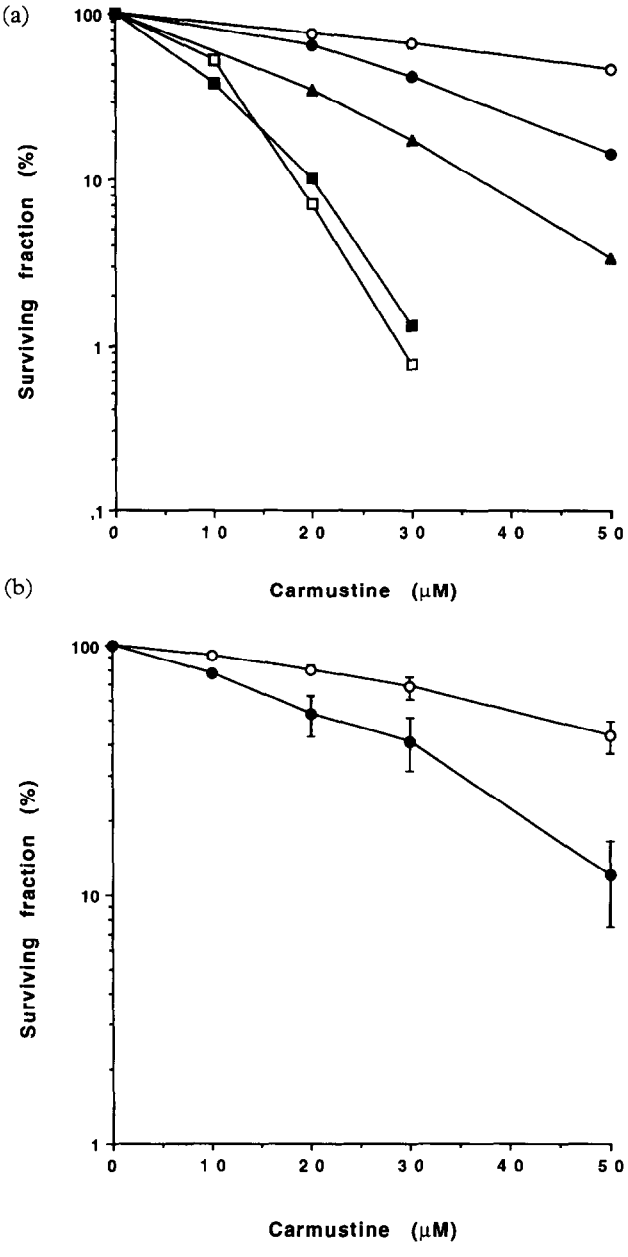

Figure 2. (a) Effect of O^6-benzylguanine on carmustine toxicity. U1810 and U1690 cells were pre-incubated for 2 h with 5 μM O^6-benzylguanine and then incubated with various concentrations of carmustine. In some cases, U1810 cells received an additional post-treatment with O^6-benzylguanine for 20 h. Symbols: U1810: no O^6-benzylguanine (○); 5 μM O^6-benzylguanine, 2 h (●); 5 μM O^6-benzylguanine, 20 h (▲); U1690: no O^6-benzylguanine (□); 5 μM O^6-benzylguanine, 2 h (■). Mean values of two separate experiments. (b) Effect of BSO on carmustine toxicity. U1810 cells were pre-incubated for 24 h with 10 μM BSO and then incubated with various concentrations of carmustine. Symbols: no BSO (○); 10 μM BSO (●). Mean values of four separate experiments, bars indicate SEM.

2-fold to carmustine (Figure 2b), a similar magnitude to that obtained by pretreatment with O^6-benzylguanine for 2 h. However, no further sensitisation to carmustine was obtained by combined treatment with BSO and O^6-benzylguanine (a dose modulating factor of two when calculated on the basis of the IC_{50} values), compared to that achieved by each agent alone.

When ethacrynic acid was added to the BSO/O^6-benzylguanine combination and used before exposure to carmustine, the toxicity was not significantly increased (data not shown), compared to the effect obtained with the BSO/O^6-benzylguanine combination only.

DISCUSSION

In the present study the intracellular levels of three putative drug-resistance factors, MGMT, GST and GSH, have been manipulated in order to determine their relative importance for resistance to the alkylating drug carmustine in the non-small lung cancer cell line U1810. We found that addition of a GST inhibitor had no detectable effect on carmustine sensitivity, whereas inhibition of MGMT and depletion of GSH had significant sensitising effects.

The finding that ethacrynic acid preferentially inhibits class mu GSTs [9] together with the demonstration that the isoenzyme GST M3-3 inactivates carmustine [11] suggested that ethacrynic acid might be a suitable agent for increasing the cell's sensitivity to carmustine. Despite the fact that U1810 cells do express the GST M3-3 isoenzyme, no potentiating effect was observed by employing ethacrynic acid. It has been shown that 5.2 μM ethacrynic acid inactivates 50% of the GST M3-3 enzyme [18]. We used 10 μM ethacrynic acid to inhibit GST M3-3, a concentration which may not have caused a sufficient inhibition of the enzyme. Higher concentrations of ethacrynic acid were not used since they are toxic to the cells. Thus, although our results indicate that GST M3-3 is of minor importance for resistance to carmustine, we cannot exclude that the lack of effect may depend on an insufficient enzyme inhibition, even though class mu GST M3-3 is more sensitive to inhibition by ethacrynic acid than are the alpha and pi class enzymes GST A1-1 and GST P1-1 [9, 18].

Our results establish the importance of both GSH and MGMT in carmustine resistance. Gerson and associates, studying the MCF-7 breast cancer cell line, found that MGMT was predominantly responsible for carmustine resistance, whereas GSH had only a small effect on the resistance to carmustine [20]. The difference in contribution of GSH to carmustine resistance found in the two investigations may depend on the relative concentrations of GSH and MGMT in the different cell types. The U1810 cells, which showed a 2-fold sensitisation to carmustine after preincubation with BSO, had approximately six times higher levels of GSH than the MCF-7 breast cancer cell line, which showed only a minor effect of BSO on sensitivity to carmustine [20].

Each resistance factor may, at varying degrees, contribute to the overall resistance in different kinds of tumours and to different kinds of drugs, depending on its relative amount in comparison to other resistance factors in the same cells. The effect of O^6-benzylguanine on the sensitivity to carmustine and dacarbazine was examined in lymphocytes and blast cells derived from patients with chronic lymphatic leukaemia (CLL), acute myeloid leukaemia (AML) or from healthy donors [21]. O^6-Benzylguanine significantly enhanced the toxicity of dacarbazine in CLL lymphocytes, but no significant effect was observed with carmustine. These results suggest that dacarbazine-induced damage is repaired to a large extent by MGMT, whereas MGMT may play a less important role in the repair of carmustine-induced damage in these cells.

In the present study, pretreatment with BSO and O^6-benzylguanine gave a similar potentiating effect on carmustine toxicity, but the two agents lacked additive effects. This may be explained by the fact that GSH and MGMT, to some extent, are competitors, since they may react with the same precursor lesions of DNA interstrand cross-links, O^6-chloroethylguanine and the rearranged form N^1-O^6-ethanoguanine. GSH quenches chloroethylated DNA, indicating that a reaction between GSH and one or both of these precursors takes place (Figure 3, II and IV) [12]. This process lowers the levels of carmustine-induced DNA interstrand cross-links (Figure 3, V). MGMT removes the O^6-chloroethylguanine monoadducts (Figure 3, I) and thereby prevents the formation of carmustine-induced DNA interstrand cross-links [1]. Furthermore, MGMT reacts with the exocyclic adduct N^1-O^6-ethanoguanine and forms a DNA–MGMT complex (Figure 3, III), a secondary DNA lesion. It has been suggested that this lesion may be subject to an excision repair process [22].

It has been shown that depletion of GSH by BSO reduces the amount of available deoxyribonucleotide triphosphates (dNTPs). This is due to GSH being involved in the synthesis of dNTPs from ribonucleotide diphosphates catalysed by ribonucleotide reductase through a system involving glutaredoxin, NADPH and glutathione reductase (GSSG-R) [23]. BSO may, therefore, have an effect on DNA repair synthesis [24].

In evaluating our results, it should be noted that carmustine by itself may contribute to GSH depletion by decreasing the levels of reduced GSH through inhibition of GSSG-R [25], an enzyme that is required for the maintenance of normal reduced GSH levels. It is the reduced form of GSH that has the detoxifying properties utilised in the conjugation of toxic agents such as carmustine. When the capacity of the cells to regenerate GSH from GSSG is depressed after carmustine treatment, an accumulation of the oxidised disulphide form will occur. An accumulation of this disulphide interferes with basic processes in the cells, and an increased export of GSSG from the cells occurs as a protective mechanism [26]. As indicated above, treatment with carmustine may also have an effect on DNA repair synthesis. Thus, besides being an alkylating agent, carmustine through inhibition of GSSG-R lowers both the level of reduced GSH and the dNTP pools.

In conclusion, the present study establishes that both GSH and MGMT are important determinants for resistance to carmustine in the U1810 cells. No clear evidence for a significant role of GSTs, in particular GST M3-3, was obtained. However, other factors may also contribute to carmustine resistance, such as nucleotide excision repair of DNA [27] and base excision repair mediated by 3-methyladenine DNA glycosylase [28], indicating that drug resistance is a multifactorial phenomenon. A few reports have presented results from investigations where several different putative resistance factors, such as MGMT, P-glycoprotein, GSH, GSTs and glutathione peroxidase, have been analysed simultaneously in tumour samples from patients [29, 30]. Individual patients showed very different patterns in the expression of these cellular components. Prior knowledge of the expression of putative resistance factors makes it possible to select drugs for treatment which may have a higher efficiency due to low levels or lack of expression of some resistance factors. In the future, prospective studies are

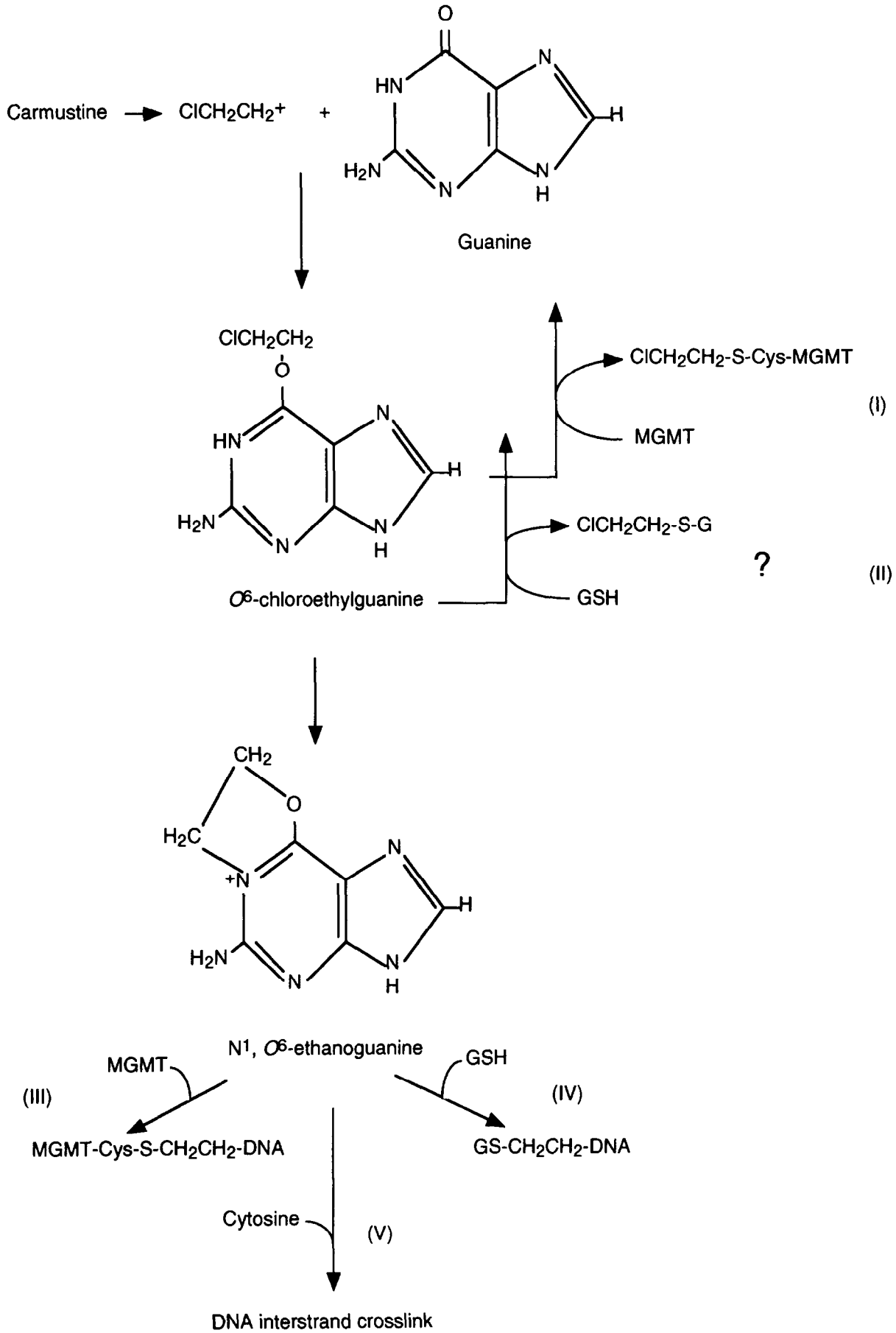

Figure 3. Proposed scheme for carmustine-induced formation of DNA interstrand cross-linking (V) as well as its inhibition by processes involving MGMT (I and III) and GSH (II and IV).

required to evaluate the clinical relevance of resistance factors for response to chemotherapy.

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Acknowledgements—This work was supported by grants from the Stockholm Cancer Society and the Swedish Cancer Society, Sweden.