[CANCER RESEARCH 49, 3525–3528, July 1, 1989]

Thermodynamic Analysis of the Reaction of Phosphoramide Mustard with Protector Thiols[1]

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ABSTRACT

The systemic use of thiol-containing uroepithelial protecting agents, *e.g.*, *N*-acetylcysteine (NAC) or mesna, in conjunction with the alkylating agent cyclophosphamide is predicated on the assumption that the toxic metabolic by-products will be consumed by thiol without diminishing the cytotoxicity of the active alkylating intermediate, phosphoramide mustard. Studies in murine tumor systems have been with either a single dose or two equally divided doses of thiol, administered within 30 min of the addition of cyclophosphamide, without an observed adverse effect on antitumor activity; however, the relatively short serum half-life of thiol relative to alkylating agent in humans weakens the clinical relevance of these results. This study presents a thermodynamic model for the chemical reaction of phosphoramide mustard with either NAC or mesna. The gas phase thermodynamic parameters for these reactions, enthalpy (H) and entropy (S), were calculated using the semiempirical quantum mechanical method AM1 and were used to predict the free energy (ΔG) for these processes. For the reaction of phosphoramide mustard with NAC or mesna, ΔG = +3.82 and 2.29 kcal/mol, respectively. In the absence of enzyme catalysis, these results suggest that such reactions are not favored. In order to assess the validity of this gas phase thermodynamic model, the cellular cytotoxicity of phosphoramide mustard in the presence or absence of either NAC or mesna was studied using CCRF-CEM cells in culture. In these experiments the 50% effective dose of phosphoramide mustard was 1.7 μg/ml; this result was unchanged in the presence of 10 μg/ml concentration of either thiol. This study supports the conclusion that phosphoramide mustard and protector thiols are compatible.

INTRODUCTION

Cyclophosphamide is inactive as an oncolytic agent and requires hepatic mixed function oxidase-mediated activation to generate the reactive cytotoxic species phosphoramide mustard (Fig. 1) (1). This compound is a bifunctional alkylating agent capable of forming DNA-protein cross-links and DNA interstrand cross-links (2–4). An unavoidable by-product of the metabolism of phosphoramide is acrolein, a reactive aldehyde thought to be the principal mediator of uroepithelial toxicity (5, 6).

NAC[3] or mesna, when employed as bladder-protecting thiols, readily combine with acrolein to yield thioethers as nontoxic adducts (7). Several studies in laboratory animals bearing a variety of transplantable tumors have concluded that protector thiols can ameliorate bladder toxicity with no apparent decrease in the antitumor activity of cyclophosphamide (7–14). In contrast to these observations are mass spectral and ^{31}P NMR studies which provide convincing spectroscopic evidence for the sequential conversion of phosphoramide mustard into mono- and bisthioether derivatives in the presence of thiols including mesna (15–17). These experiments support the view that phosphoramide mustard undergoes alkylation as an intact molecule via an intermediate aziridinium ion.

There is accumulating *in vitro* evidence that one aspect of tumor resistance to alkylating agents such as cyclophosphamide involves increased intracellular levels of glutathione and glutathione-*S*-transferase activity (18). In this model, glutathione-*S*-transferase catalyses an irreversible reaction between the thiol glutathione and the alkylating agent to give a thioether which is devoid of alkylating ability.

The direct and enzyme-mediated alkylation of phosphoramide mustard by thiols raises the concern that the cytotoxicity of cyclophosphamide may be sacrificed in the presence of protector thiols. This report describes the use of AM1, a semiempirical theoretical model, to compute the change in free energy on reaction of phosphoramide mustard with NAC or mesna. The validity of this model is assessed by incubation of CCRF-CEM cells with phosphoramide mustard in the presence and absence of NAC or mesna.

MATERIALS AND METHODS

Chemicals. NAC was purchased from Aldrich Chemical Co. Mesna (sodium 2-mercaptoethane sulfonate) was obtained from the National Cancer Institute (Bethesda, MD). Phosphoramide mustard, cyclohexylamine salt, was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

Computational Studies. Geometry calculations were performed on a VAX 8800 Series computer and thermodynamic calculations were performed on a Cray XMP-48. Starting geometries for all molecules were generated in the molecular modeling environment Macromodel[4] and were energy minimized using the Block Diagonal Newton-Rafson algorithm and the molecular mechanics force field MM2 (19). The resultant set of atomic coordinates was transferred to MOPAC using the AM1 Hamiltonian system (20) and the geometries were further optimized. Thermodynamic parameters were calculated for 310 K at convergence using AM1 in the precise mode (double-precision variables).

CCRF-CEM Cytotoxicity Assay. CEM cells were grown in RPMI 1640 medium (MA Bioproducts) that contained 10% dialyzed fetal bovine serum (GIBCO), at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. The cells were maintained in static suspension (T-flask) in log growth phase at a concentration of 3×10^5 cells/ml. Phosphoramide mustard was dissolved in saline and sterile filtered through a 0.2-μm filter (Gelman). The phosphoramide mustard solution (10 μl) and 400 μl of phosphate-buffered saline were transferred to each well of a 24-well Costar cluster. Cells were transferred to the wells from a magnetically stirred suspension that contained 3×10^4 cells/ml in RPMI 1640 medium with 10% fetal bovine serum, 8 mM 3-[*N*-morpholino]propanesulfonic acid, and 16 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffers. The resulting volume was 2.0 ml, with a concentration of 4.8×10^4 cells/well. The cluster plates were incubated as above for 72 h, at which time the cells were read using a model ZBI Coulter particle counter. For experiments requiring the coadministra-

Received 12/5/88; revised 3/27/89; accepted 4/4/89.

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[1] Presented in part at the 1988 Meeting of the American Association for Cancer Research.

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[3] The abbreviations used are: NAC, *N*-acetylcysteine; NMR, nuclear magnetic resonance; ED_{50}, 50% effective dose.

[4] The computer program, Macromodel, used in these calculations, is available from Prof. W. C. Still, Department of Chemistry, Columbia University (New York, NY).

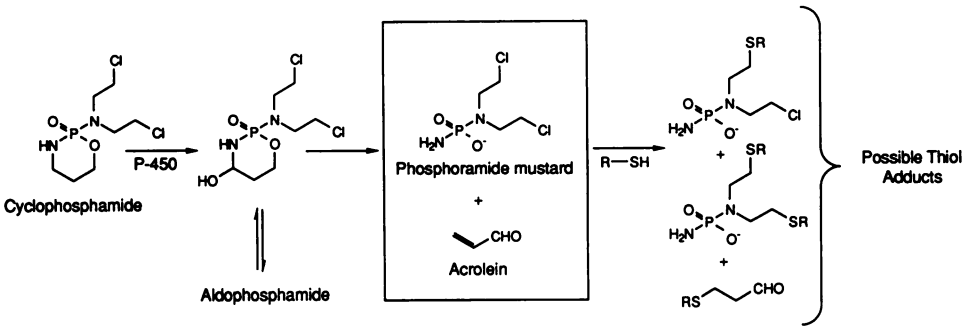


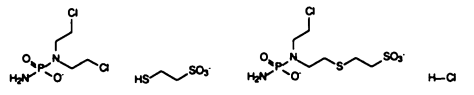
Fig. 1. Metabolic conversion of cyclophosphamide to the products phosphoramide mustard and acrolein and their subsequent conversion, on reaction with thiols, to thioether adducts.

tion of thiol, solutions of either NAC or mesna in saline sufficient to adjust the final concentration of thiol in the medium to 10 μg/ml were added to the culture every 8 h for the duration of the growth study.

RESULTS

The gas phase thermodynamic parameters enthalpy (H) and entropy (S) were calculated for each component (reactants and products) of the reaction of phosphoramide mustard with either NAC or mesna, using AM1. The change in free energy (ΔG) of the reaction was derived using the equation $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature (T = 37°C = 310 K) and ΔH and ΔS are the differences in the sums of H and S for the reactants and products, respectively. For the reaction of phosphoramide mustard with NAC (Fig. 2*A*), ΔG = +3.82 kcal/mol and, for the reaction of phosphoramide mustard with mesna (Fig. 2*B*), ΔG = 2.29 kcal/mol.

A



	PAM	Mesna	PAM-Mesna	H—Cl
Enthalpy (H, cal/mol)	9630.	6796.	15291.	2156.
Entropy (S, cal/K/mol)	128.	99.4	178.4	44.9
Free Energy (G, cal/mol)	-30050.	-24,018.	-40,013.	-11,763.

For the reaction:

PAM + Mesna ---------> PAM-Mesna + HCl

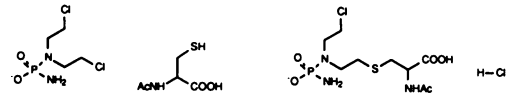
ΔG = [GPAM-Mesna + GHCl] - [GPAM + GMesna]

ΔG = [(-40,013.) + (-11,763.)] - [(-30,050.) + (-24,018.)]

ΔG = -51,776. - (-54,068.)

ΔG =2,292. cal/mol

B



	PAM	NAC	PAM-NAC	H—Cl
Enthalpy (H, cal/mol)	9630.	8,740.	17,211.	2156.
Entropy (S, cal/K/mol)	128.	117.	191.0	44.9
Free Energy (G, cal/mol)	-30,050.	-27,530.	-41,999.	-11,763.

For the reaction:

PAM + NAC ---------> PAM-NAC + HCl

ΔG = [GPAM-NAC + GHCl] - [GPAM + GNAC]

ΔG = [(- 41,999.) + (- 11,763.)] - [(- 30,050.) + (- 27,530.)]

ΔG = - 53,762. - (- 57,580.)

ΔG = 3,818. cal/mol

Fig. 2. Calculated thermodynamic parameters for all components of the reaction of phosphoramide mustard (*PAM*) with protector thiols to form monothioether adducts. *A*, reaction of phosphoramide mustard with NAC; *B*, reaction of phosphoramide mustard with mesna.

The cellular cytotoxicity of phosphoramide mustard in the presence or absence of either NAC or mesna was determined using CCRF-CEM cells in culture. ED_{50} values were interpolated from dose-response curves (Fig. 3) that were generated by varying the concentration of phosphoramide mustard at constant thiol concentration. In order to ensure that reactive thiol was present, a fresh aliquot of thiol solution was added to the medium every 8 h for the duration of the incubation period. The EC_{50} of phosphoramide mustard was 1.7 μg/ml; this result was unchanged in the presence of 10 μg/ml concentrations of either thiol.

DISCUSSION

There are conflicting data on the compatibility of phosphoramide mustard and thiol. As early as 1976, Colvin *et al.* (15) provided unequivocal mass spectral evidence for the formation of a bisthioether adduct from the reaction of phosphoramide mustard and aqueous ethanethiol at physiological pH. Analysis of the reaction mixture was consistent with alkylation proceeding via an intermediate aziridinium ion rather than direct (S_N2) displacement of chloride by thiol.

The products and reaction kinetics of the decomposition of phosphoramide mustard in the presence of nucleophilic trapping agents have been evaluated utilizing ^{32}P Fourier-transform NMR spectroscopy (16, 17). The cascade of reactions leading to intermolecular alkylation of phosphoramide mustard *versus* competing nitrogen-phosphorus bond scission is strongly pH dependent. At 37°C the half-life of phosphoramide mustard is nearly constant (18 ± 3 min) between pH 7.0 and 9.0. Over this pH range, formation of an aziridinium ion by intramolecular displacement of chloride and subsequent ring opening by nucleophile was observed, whereas at pH values ≤ 6.5, hydrolysis of the nitrogen-phosphorus bond is the predominant reaction. When an excess of mercaptoethanol or mesna was used as the trapping agent, separate ^{31}P NMR signals for the monothioether and bisthioether products were recorded. Analysis

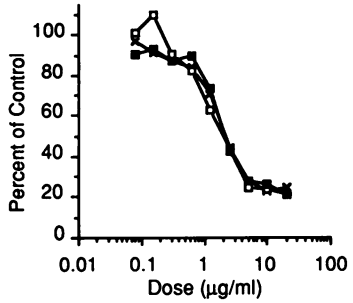


Fig. 3. Dose-response relationship of the effect of phosphoramide mustard on the growth of CCRF-CEM cells in culture, alone (×) and in the presence of either NAC (■) or mesna (□) at a concentration of 10 μg/ml.

of these spectral data gave rate constants of 0.036 and 0.043 min^{-1} for formation of the monothioether and bisthioether derived from mercaptoethanol, respectively.

In contrast to these chemical observations are studies in experimental tumor models, wherein several authors have reported that NAC does not cause a significant decrease in the antitumor activity of cyclophosphamide while preventing acrolein-induced hemorrhagic cystitis and depression of hepatic mixed function oxidase (7–11). In these experiments, NAC was administered either as a single dose or as two equally divided doses by a variety of routes (i.p., p.o., or i.v.) within 30 min of the addition of cyclophosphamide, in a ratio which ranged from 1300:1 to 1:1 NAC:cyclophosphamide.

In similar fashion, mesna administered i.p. 20 min prior to the addition of cyclophosphamide (0.3:1 to 1:1 mesna:cyclophosphamide) provided effective uroepithelial and hepatic mixed function oxidase protection with no reduction in antitumor effectiveness, in a variety of transplantable tumors (7, 8, 12, 13). In fact, in C57BL/6 mice bearing B16 melanoma or M5076 sarcoma, a small improvement in life span was noted in the presence of mesna.

The relative levels of glutathione and the enzyme glutathione-*S*-transferase have been associated with increased cellular resistance to a variety of alkylating agents (18). Glutathione exerts schedule-dependent protective effects on the acute lethal toxicity of cyclophosphamide and on acrolein-induced uroepithelial damage without sacrificing cyclophosphamide cytotoxicity, in experimental tumor systems (14). In these studies glutathione was administered in a fractionated i.v. dose 30 min before and 30 min after an i.p. dose of cyclophosphamide. The ratio of glutathione to cyclophosphamide varied from 1:1 to 5:1.

The *in vivo* studies which have examined the compatibility of chemoprotective thiols and cyclophosphamide are characterized by the addition of thiol within 30 min of the administration of cyclophosphamide. The markedly shorter half-life of these thiols relative to cyclophosphamide indicates that the metabolism of cyclophosphamide continues in the absence of thiol (21, 22). In addition, the inefficient cellular uptake of exogenously administered thiol suggests that the original conclusion, that thiols do not diminish the cytotoxicity of cyclophosphamide, may be in error, particularly if the weakly electrophilic metabolite 4-hydroxycyclophosphamide is responsible for the transport of phosphoramide mustard into the cell (23–26).

The probability that a chemical reaction will proceed is determined by a combination of kinetic and thermodynamic parameters. Reactions that are controlled predominantly by kinetic effects are characterized by relatively low activation barriers that are overcome by the energetics of the reactants, and the composition of the reaction products is determined by the relative rates of competing reactions. In contrast, reactions that proceed under thermodynamic control are characterized by a large negative difference in the combined free energies of the reaction products, relative to those of the reactants. The composition of the reaction products is determined at equilibrium. The majority of chemical reactions fall between these extremes and are controlled by a combination of these effects (27).

In order to understand the obvious dichotomy between the reported thiol-alkylating properties of phosphoramide mustard and the insensitivity of the cytotoxic and antitumor properties of cyclophosphamide to added thiol, a thermodynamic analysis of the reaction of phosphoramide mustard with NAC or mesna was undertaken. The enthalpy and entropy for each reactant and product in the balanced reaction of phosphoramide mustard with these thiols were calculated using the semiempirical quantum mechanical model AM1, and these parameters were used in turn to calculate the free energy for each component. A comparison of the sums of the free energies of the reactants and products gave a large positive value for both reactions (Fig. 2), indicating that these reactions are not thermodynamically favored.

This theoretical analysis may be compromised by certain features. (*a*) The treatment describes a gas phase reaction. The computational complexities inherent in calculating solvation energies preclude the inclusion of these effects necessitating the use of charged species in these calculations. (*b*) The treatment ignores kinetic parameters for the formation of an intermediate aziridinium ion and the subsequent reaction with nucleophilic thiol. Thus, it is impossible to assess the importance of enzyme catalysis in delivering thiol to the electrophilic acceptor; however, the large positive ΔG determined for these processes indicates that enzyme catalysis may be required.

Studies on the thiol-alkylating properties of phosphoramide mustard have primarily used the neutral thiols 2-mercaptoethanol and ethanethiol. The nucleophilic properties of these thiols relative to phosphoramide mustard may differ from those of the charged thiols NAC and mesna, and this difference may contribute to the discrepancies noted above. In order to study the effect of NAC and mesna on the cytotoxicity of phosphoramide mustard, CCRF-CEM cells were incubated in the presence of high concentrations of either NAC or mesna with increasing concentrations of phosphoramide mustard (Fig. 3). In these experiments the medium was replenished with thiol every 8 h for the duration of the 72-h incubation period. There was no change in the observed ED_{50} of phosphoramide mustard (1.7 μg/ml) in the presence of thiol.

The cell culture studies are in agreement with the thermodynamic analysis of the reaction of phosphoramide mustard with charged thiols, and these observations suggest that enzyme catalysis (namely, glutathione-*S*-transferase) may be operative *in vivo*. This premise has not been established and requires further evaluation.

ACKNOWLEDGMENTS

The authors wish to gratefully acknowledge Cray Research (Mendota Heights, MN) for making available supercomputing facilities; Dave Smith, Lilly Research Laboratories, for assistance in transmitting large data files; and Dr. Donald B. Boyd for making available MOPAC command procedures.

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