Relative Mutagenicity of Some Urinary Metabolites of the Antitumor Drug Cyclophosphamide

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ABSTRACT

Four urinary metabolites of the cytostatic drug cyclophosphamide were tested for mutagenicity in the Ames Salmonella assay: nor-nitrogen mustard (NM), 4-ketocyclophosphamide, 3-(2-chloroethyl)oxazolidone (OZ), and N,N'-bis(2-chloroethyl)piperazine. All four acted as direct base substitution mutagens although 4-ketocyclophosphamide showed an increase in mutagenicity after metabolic activation with S-9 rat liver fraction. Of the four compounds tested, NM was the strongest mutagen while all the others had weak mutagenic activity, with OZ being the weakest. We observed that, under conditions which facilitate the conversion of NM to OZ (presence of HCO$_3^-$ at neutral pH), the former lost both mutagenic and alkylating activities. Our findings, taken together with other reports in the literature, indicate that NM could be a major cause of secondary bladder carcinoma since it is a potent mutagen and seems to be present in high levels in the urine of cyclophosphamide-treated patients. The fact that it can be detoxified to the weak mutagen OZ in the presence of HCO$_3^-$ suggests the possibility that, by increasing the concentration of HCO$_3^-$ in the urine of patients, that undesirable side effect of cyclophosphamide treatment can be alleviated.

INTRODUCTION

The drug CY is used extensively as an antineoplastic agent for the treatment of various cancers and also as an immunosuppressive agent for certain nonmalignant conditions (16). In recent years, some complications in CY therapy have been reported. The drug has been implicated in producing both primary and secondary cancers in animals and humans, including both leukemias and solid tumors (28, 29, 36). Several of the side effects of CY treatment affect the urinary tract and include sterile hemorrhagic cystitis (2), as well as the more serious condition of urinary bladder carcinoma (37). CY has been shown to be a mutagen (4, 20, 25-27, 30, 31, 34) and a carcinogen following activation by microsomal liver enzymes (6, 21-24), a knowledge of the relative mutagenicities of the urinary metabolites of CY could help in identifying the causative agent(s) of secondary urinary bladder carcinoma.

MATERIALS AND METHODS

Mutation Test. Mutagenesis tests were carried out on the standard Salmonella his strains of Ames et al. (1), TA98, TA100, TA1535, TA1537, and TA1538, which were kindly supplied by Dr. Ames. We used the plate incorporation assay described in the reference above as modified by Yahagi et al. (40), according to the following protocol. Tester bacteria (100 µl) from a fresh overnight broth culture were mixed with 100 µl of a desired concentration of mutagen in a sterile 13- × 100-mm test tube, and the mixture was allowed to stand at room temperature for 20 min. When metabolic activation was desired, 500 µl of metabolic activation mixture (1) were added. This was substituted by 500 µl of 100 mM sodium phosphate buffer (pH 7.4) when activation was not required. For negative controls, the mutagen was replaced by 100 µl of dimethyl sulfoxide. Thus, the final volume of the preincubation mixture was always 700 µl, except when specified. After 20 min, 2.5 ml of molten top agar (0.6% Bacto-Difco agar containing 0.05 mM histidine and 0.05 mM biotin) were added to each tube. The mixture was stirred gently and poured on top of a minimal agar plate. Treatment for the biological test was with 100 µl of preincubated mixture as above. Briefly, 50 µl of a nutrient broth culture of Salmonella his strain TA100 were added to each plate and incubated at 37°C for 48 h. The plates were scored for colonies, and the results were expressed as the number of colonies on the plates divided by the number of colonies on the untreated controls. The results were considered significant (P < 0.05) if the number of colonies on the treated plates exceeded that on the control plates by at least 2-fold or if the number of colonies was reduced by at least 2-fold. The results are given in Table 1.

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Chart 1. Metabolism of CY. CY is first oxidized at C-4 via a microsomal cytochrome P-450-linked enzyme system in the presence of NADPH and molecular oxygen to 4-hydroxycyclophosphamide. This initial product equilibrates with its acyclic tautomer, aldophosphamide, which is further converted by a soluble aldehyde oxidase to carboxyphosphamide, which is found in urine. Another metabolic step leads by decomposition of aldophosphamide to 2 cytotoxic metabolites, AC and PM. 4-Hydroxycyclophosphamide is acted upon by an oxidase to form KCP which is found in the urine. NM seems to originate from the spontaneous breakdown of PM and carboxyphosphamide.

Chart 2. Conversion of NM to 2 metabolites found in urine. NM is converted to OZ in aqueous solution in the presence of HCO₃⁻ at neutral pH through a carbamate ion intermediate (CPD A). The dimerization of NM to PZ could happen as shown in the chart, but it is not certain that this reaction takes place in vivo (10).

glucose agar plate. The top agar was allowed to set for a few min at room temperature, and the plates were then incubated at 37° for 2 days in the dark. After incubation, the number of his⁺ revertant colonies was counted with an automatic plate counter and recorded. This counter is routinely calibrated against hand counts as recommended by DeSerres and Shelby (11). The S-9 rat liver homogenates for metabolic activation were prepared by the procedure of Ames et al. (1), with the difference that we used Holtzman instead of Sprague-Dawley rats. We followed the recommendations of Ames et al. (1) and DeSerres and Shelby (11) in our choice and use of standard control mutagens.

Chemicals. CY was from Mead Johnson Laboratories, Evansville, Ind. The various metabolites of CY were synthesized just before use by the procedures described below. They were found to be at least 98% pure by gas chromatography, thin-layer chromatography, mass spectrometry, and elemental analysis.

KCP was prepared according to the method of Hill et al. (17) and Jarman (19) by the chemical oxidation of CY with KMnO₄ or with Fenton's reagent (H₂O₂, H₂SO₄, Fe²⁺). The product was isolated as a white crystalline solid from ether:ethanol, m.p. 149-150° (literature, 150°). NMR (acetone-d₆): δ 2.68 (m, 2H, CO—CH₂); 3.58 ppm [m, 8H, (CH₂—CH₂—Cl)₂]; and 4.35 ppm (m, 2H, CH₂—O). IR (KBr): 3500 (N—H) and 1710 cm⁻¹ (C=O).

OZ was prepared in 86% yield by the reaction of bis(2-chloroethyl)amine hydrochloride (Aldrich Chemical Co., Milwaukee, Wis.) with potassium bicarbonate as reported by Williamson et al. (39) and Arnold and Bekel (3). The product was isolated as a pale yellow oil by Kugelrohr bulb-to-bulb distillation at 90°/0.05 mm Hg (literature, 116-117°/0.35 mm Hg). NMR (CDCl₃): δ 3.8 (m, 6H, H₂C—N—CH₂—CH₂—CD and 4.32 ppm (t, 2H, COO—CH₂—). Mass spectrum: m/e, 151 (μ⁺, CI₃7, 6%); 149 (μ⁺, CI₃5, 17%); 100 (100%); 86 (25%); and 84 (34%).

NM was obtained as a colorless oil by treatment of its hydrochloride salt (Aldrich Chemical Co.) with dilute sodium hydroxide followed by extraction with chloroform, drying (Na₂SO₄), filtration, and short-path distillation. NMR (D₂O): δ 3.50 ppm (t, 4H, N—CH₂—CH₂—CD and 3.90 ppm (t, 4H, N—CH₂—CH₂—CI).

PZ was prepared by the procedure of Childs et al. (7). N,N'-bis(2-hydroxyethyl)piperazine (15 g; 0.086 mol) was suspended in dimethylformamide (200 ml) followed by the drop-wise addition of thionyl chloride (25 g; 0.21 mol). The reaction mixture was heated to reflux until gas evolution ceased, cooled to room temperature, poured into chloroform (750 ml), and cooled to −10° for 12 hr. The cold solution was filtered, and the precipitate was dissolved in water (75 ml) which was made basic with NaOH and extracted with methylene chloride. The methylene chloride solution was dried (Na₂SO₄) and filtered, and the solvent was removed to yield the crude product (16.8 g; 92%) as a tan solid. A short-path vacuum distillation (100°/0.15 mm) yielded the pure product as a white solid, m.p. 38-40° (literature, 41-43°), the structure of which was confirmed by spectroscopic methods.

Chemical Determination of Alkylating Activity. The alkylating capacities of NM and OZ were determined by the colori-
metric method of Friedman and Boger (15) using NBP. This was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The mutagenic potentials of the various CY metabolites included in this study were first tested against all the standard Salmonella his strains, both in the presence and absence of S-9. The results are shown in Table 1. It is clear that positive responses were obtained only with strains TA1535 and TA100 which have the base substitution mutation hisG46 (1). Thus, in agreement with the results of other workers (4, 12, 30, 34), CY and its metabolites can cause base substitutions but not frameshifts. In addition, while CY requires metabolic activation to show any detectable mutagenic activity, all the other metabolites were direct mutagens. This also had been reported previously (12). One new observation is that KCP, which can act as a direct mutagen, showed an increase in mutagenic activity in the presence of S-9 (Chart 3). This is not surprising since KCP was the weakest mutagen of all the CY metabolites tested by Ellenberger and Mohn (12), and it is conceivable, considering its structure (Chart 1), that it could be degraded by the mixed-function oxidase system of the liver to the more mutagenic compounds further down in the pathway. In this experiment, the effective concentrations of KCP in 700 μl of preincubation mixture ranged from 5.2 (1 mg/plate in Chart 3) to 155.8 mM (30 mg/plate in Chart 3).

Chart 4 summarizes the results of several experiments comparing the direct mutagenic potentials of NM, KCP, OZ, and PZ. It is clear that, of the metabolites tested previously by Ellenberger and Mohn (12), NM is by far the strongest mutagen and KCP is weakly mutagenic, in agreement with their observations. Both of the previously untested metabolites, OZ and PZ were very weak mutagens, with the latter being perhaps a little weaker than KCP and the former clearly weaker than both KCP and PZ. Thus, of all the urinary metabolites of CY, OZ has the weakest mutagenic potential. Arnold and Bekel (3) showed that NM is converted to OZ in the presence of NaHCO₃ at neutral pH and speculated that this could be correlated with its observed loss of cytotoxic activity. Since our results in Chart 4 show that OZ is much less mutagenic than NM, we would expect that the latter will lose mutagenicity under conditions such as those shown by Arnold and Bekel (3) to facilitate its conversion to the OZ. To test this idea we performed an experiment where 2 concentrations of HCO₃⁻ were added to the standard mixture of bacteria and mutagen and preincubated for 20 min according to the protocol described under "Materials and Methods." As shown in Chart 5, increasing amounts of NM ranging from 10 to 100 μg/plate were used. This corresponds to an effective concentration range of 0.1 to 1.0 mM in the 700 μl of preincubation mix. The results of this experiment are presented in Chart 5 and show that NM did indeed lose mutagenicity in proportion to the concentration of HCO₃⁻ to which it was exposed. Chart 6 presents the results of an experiment designed to determine the rate of loss of the mutagenic activity of NM on exposure to HCO₃⁻. In this experiment, 100 μl of a 7 mM solution of NM were preincubated with 500 μl of 100 mM phosphate buffer, pH 7.4, with and without 50 mM NaHCO₃, for different periods of time as indicated. After preincubation, 100 μl of an overnight culture of TA1535 were added to each tube, and the mixture was incubated for 20 min, mixed with melted agar, and poured onto 2% minimal glucose plates, as per the standard protocol (see "Materials and Methods"). As shown in Chart 6, there was an immediate drop in the mutagenicity of NM and no further change for the remainder of the preincubation period. This indicates that the conversion of NM to OZ is very rapid. In agreement with this observation, we found during the synthesis of OZ with bicarbonate (see "Materials and Methods") that this reaction proceeded at very high speed. It was not possible to estimate its kinetics by NMR since immediately after addition of NaHCO₃ to a sample of NM, only OZ was detected. A stored solution of NM soon crystallized OZ presumably as a result of the reaction of NM with CO₂ in the air, and only storage under an inert atmosphere with complete exclusion of CO₂ prevented this reaction from taking place.

Considering the rapidity of the reaction, we would expect

![Chart 3. Mutagenicity of KCP to strain TA1535 in the presence (○) and absence (○) of S-9. The results have been corrected for spontaneous revertants (15/plate).](image-url)
that, under conditions where HCO$_3^-$ was present in high excess over NM, the mutagenicity of the latter would be completely or almost completely abolished, but this did not seem to be the case under the conditions we used. In the experiment summarized in Chart 5, the effective concentrations of NM ranged from 0.1 to 1.0 mM while the concentrations of HCO$_3^-$ were 10 and 100 mM. This means that, in the preincubation volume of 700 µl, at the lowest NM concentration, we had a total of 0.07 µmol of the mutagen and at the highest 0.7 µmol. For HCO$_3^-$, the effective concentrations were, respectively, 7 µmol (for the 10 mM series) and 70 µmol (for the 100 mM series). The HCO$_3^-$:NM molar ratios thus ranged from 10 to 1000. For the experiment summarized in Chart 6, HCO$_3^-$ and NM were preincubated in a total volume of 600 µl prior to the addition of the bacteria. In this volume, we had a total of 30 µmol of HCO$_3^-$ to 0.7 µmol of NM, a molar ratio of 43. In summary, although in these 2 experiments the ratios of HCO$_3^-$ to NM varied over a 100-fold range, the observed decrease in mutagenicity was only between 2- and 4-fold. We will return to this after considering the next experiment.

A good correlation between mutagenic and alkylating potentials has been shown for NM and other CY metabolites (12). We can expect then that the loss of mutagenicity of NM that we observed on treatment of this compound with HCO$_3^-$ (Charts 5 and 6) will be paralleled by a decrease in its alkylating ability as measured in the NBP reaction (15). This was indeed the case, as shown in Chart 7. In this experiment, aliquots of NM ranging from 5 to 100 µg in 100 µl of dimethyl sulfoxide were introduced into screw-capped 12-ml round-bottomed tubes in a final volume of 1.5 ml of 100 mM phosphate buffer, pH 7.4, with and without 50 mM HCO$_3^-$, and put on ice. To each test tube were then added 500 µl of 200 mM acetate buffer (pH 4.6) and 200 µl of NBP reagent (prepared to 5% in acetone). The mixture was placed in a boiling water bath for 20 min and cooled, and 1.0 ml of acetone, 2.5 ml of ethyl acetate, and 750 µl of 0.25 N sodium hydroxide were added successively. Each test tube was shaken 20 times and centrifuged for 2 min, and the absorbance of the supernatant fraction at 540 nm was determined in a Hitachi-Perkin Elmer Spectrophotometer. The last steps (from introduction of sodium hydroxide to the reading in the spectrophotometer) were carried out rapidly (within 5 min) and in the absence of light, because of the instability of the color formed after alkylization. In this test, OZ showed no detectable alkylating potential, in good...
correlation with its very weak mutagenicity. We did not test PZ for its alkylating ability. In this experiment, the effective concentrations of NM in the 1.5-mi incubation mixture ranged from 2.3 X 10^{-2} \text{mM} (at 5 \mu \text{g NM}) to 4.7 X 10^{-1} \text{mM} (at 100 \mu \text{g NM}). Thus, the molar ratios of HCO_3^- to NM ranged from about 2000 to about 100, respectively, a 20-fold variation, yet the loss of alkylating ability of NM as manifested by decrease in absorbance at 540 nm (Chart 7) was by a constant factor of between 2- and 3-fold.

In summary, our results indicate that HCO_3^- treatment of NM results in a rapid loss of the mutagenicity of the latter compound and that this loss correlates well with a decrease in the alkylating potential of the compound. However, the loss was never complete, at least under our experimental conditions. Mutagenicity and alkylating ability never decreased by a factor of more than 2 to 4, regardless of how large the excess of HCO_3^- to NM was. Possible explanations will be considered in the discussion.

DISCUSSION

Our results, taken together with other reports, lead to the conclusion that, of all the urinary metabolites of CY, NM is clearly the most mutagenic, has the highest alkylating activity, and is very cytotoxic to animal cell lines, as well as bacteria, while OZ is the weakest mutagen, has no alkylating potential, and is weakly cytotoxic (Refs. 8, 10, 12, 32, and 35 and our results). The remaining compounds are not only weaker mutagens and alkylating agents than NM (12, 35) but, with the exception of PZ and AC, are also less cytotoxic (18, 32). PZ has been reported to be about 35 times more cytotoxic than NM to Walker ascites tumor cells (10), while AC was shown to be extremely toxic to E. coli 343/133 (12) and has been implicated as the causative agent of bladder cystitis (9). As we show in this report, NM rapidly loses some of its mutagenicity and alkylating potential under conditions which are known to facilitate its conversion to OZ, namely exposure to HCO_3^- at neutral pH. Cox and Levin (10) consider OZ as a detoxification product of NM, a conclusion in agreement with our observations.

The levels of NM found in the plasma and urine of both mice and humans receiving CY have been reported to be high (10, 18, 33). Although some of this NM could be an artifact arising from the decomposition of CY, KCP, PM, and particularly carboxyphosphamide during the processing of samples, Jardine et al. (18) believe that a similar decomposition occurs in vivo and that much NM is actually present in the body, particularly in the urine. Williamson et al. (39) have reported that the reaction of NM with HCO_3^- to OZ can proceed without a catalyst although an enzyme seems to carry this out in blood plasma. The ease of the reaction and the levels of HCO_3^- in plasma strongly suggest to Cox and Levin (10) that at least some of the NM is converted to OZ in vivo and excreted as such. Not much is known at this time about PZ. Cox and Levine (10) have found evidence of its presence in human urine but are not certain that it is actually formed in vivo.

In spite of its high mutagenicity, cytotoxicity, and alkylating activity, NM seems to make no contribution to the therapeutic potential of CY. The favored candidate in this respect is PM (8, 18, 33). It is very likely, on the other hand, that NM is a major contributor to the undesirable side effects of CY treatment. Its high mutagenicity and its presence in high levels in the urine of patients treated with that drug implicate it as a possible causative agent of urinary bladder carcinoma. All the information available at this time, which we have just reviewed, points to the conclusion that NM is a highly undesirable by-product of CY metabolism. The fact that it can be detoxified to the (seemingly) harmless OZ raises the possibility that a course of treatment which would facilitate this reaction (perhaps by increasing the level of HCO_3^- in urine) might help prevent the occurrence of secondary urinary bladder carcinoma and alleviate some of the other complications of CY treatment as well.

Such a course of treatment could, perhaps, be similar to that used in the management of cystine and uric acid stones, which uses acetazolamide (an inhibitor of carbonic anhydrase) together with sodium bicarbonate (14) to bring about an increased excretion of HCO_3^- and an alkaline urine. Whether this approach can succeed would depend on how close to completion the conversion of NM to OZ can proceed in urine.

Our results show that, at least under our experimental conditions, a large excess of HCO_3^- was not sufficient to bring about more than a 2- to 4-fold drop in the mutagenicity of NM. A consideration of the behavior of NM in aqueous media (39) might help to explain this observation. Under these conditions, secondary nitrogen mustards, such as NM, cyclize to form ethylenimines which are very active alkylating agents in the charged (protonated) form. For this reason, the secondary amine mustards are generally more reactive at acidic pH, where the concentration of the protonated form is higher. At higher pH, nucleophilic ions act upon the aziridinium ion to convert it to an uncharged form. The latter, as well as the uncyclized NM, are poor alkylating agents as compared to the cyclized protonated form. HCO_3^-, when present in the medium, would not only react with uncyclized NM but could also speed up the conversion of the protonated to the unprotonated form. Thus, we are dealing, in effect, with 2 potential reactions and not one, the relative rates of which could be differently affected by a variety of factors. The detoxification of NM to innocuous compounds in aqueous media is, apparently, not a simple reaction. A better understanding of the kinetics of this reaction(s), particularly under conditions which are likely to exist in urine, seems desirable before the course of treatment suggested above can be seriously considered.

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