Oxidative Metabolism of 1-(2-Chloroethyl)-3-alkyl-3-(methylcarbamoyl)triazenes: Formation of Chloroacetaldehyde and Relevance to Biological Activity

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(Methylcarbamoyl)triazenes have been shown to be effective cancer chemotherapeutic agents in a number of biological systems. Because of their chemical stability, it is likely that their activity in vivo is the result of a metabolic activation process. Previous studies have shown that 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)triazene (CMM) and 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene (CBzM) are metabolized by rat liver microsomes in the presence of NADPH to yield the ((hydroxymethyl)carbamoyl)triazene analogs of the parent compounds. The present studies show that both compounds are also oxidized at the chloroethyl substituent to yield chloroacetaldehyde and a substituted urea. In the case of CBzM metabolism, 47% of the metabolized parent compound was recovered as benzylmethylurea, 8% was recovered as benzylurea, and 26% was recovered as the ((hydroxymethyl)carbamoyl)-triazene and carbamoyltriazene metabolites. These results suggest that the chloroethyl group is the favored initial site of metabolism. In reaction mixtures containing intitial concentrations of 300 μM CBzM, 78 μM chloroacetaldehyde was produced, as compared to 58 μM chloroacetaldehyde produced from the metabolism of 300 μM CMM. The formation of chloroacetaldehyde, a known mutagenic DNA alkylating agent, may explain the biological activity of these compounds.

Introduction

The synthesis and chemical decomposition of 1,3-dialkyl-3-acyltriazenes have been extensively studied in our laboratory (*1*−*5*). These compounds were originally designed with the expectation that they would be DNA alkylating agents through the formation of an alkanediazonium ion intermediate. As predicted, under acidic and neutral conditions, the acyltriazenes undergo heterolysis of the N(2)−N(3) bond to yield the expected alkanediazonium ion bearing the substituent originally at N(1) (*4*). Consequently, a series of 1,3-dimethyl-3-acyltriazenes and 1-(2-chloroethyl)-3-methyl-3-acyltriazenes have been synthesized to act as methylating and chloroethylating agents, respectively (*1, 2*). Biological studies revealed that all of these triazenes possessed cytotoxic activity in the MTT-microculture tetrazolium assay, with the (2-chloroethyl)triazenes generally showing higher potency than the dimethyltriazenes (*3*). In vivo, several of the compounds were also found to be active against selected tumor xenografts implanted in nude mice. Of particular interest were the findings that 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)triazene (CMM)[1] possessed remarkably low toxicity while effecting cures in the cases of the P388 murine leukemia and the LOX melanoma (*3*).

These initial studies suggested that the acyltriazenes might be a promising new class of cancer chemotherapeutic agents. However, it was soon realized that, because of their very high chemical stability under physiological conditions, their biological activity could not easily be explained by chemical decomposition alone. For example, the estimated half-life of CMM at pH 7.5 and 37 °C is between 2 and 3 days (*4*). The possibility was therefore considered that a metabolic activation step might lead to more rapid chemical breakdown. Consistent with this hypothesis were findings that acyltriazenes bearing a carbethoxy substituent demonstrated a marked increase in their ability to alkylate DNA if porcine liver esterase was included in the reaction mixtures. The esterase catalyzed the deacylation of the triazene, yielding a highly unstable dialkyltriazene which then decomposed to form an mixture of alkanediazonium ions (*6*−*8*). The mechanism of action of carbethoxytriazenes in vivo could therefore easily be explained; however, the

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[1] Abbreviations: 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)-triazene (CMM), 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene (CMC), 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene (CBzM), 1-(2-chloroethyl)-3-benzyl-3-((hydroxymethyl)carbamoyl)triazene (CBzM-met1), 1-(2-chloroethyl)-3-benzyl-3-carbamoyltriazene (CBzM-met2), 1-(2-chloroethyl)-3-methyl-3-((hydroxymethyl)carbamoyl)triazene (CMM-met1), (dinitrophenyl)hydrazone of chloroacetaldehyde (CAA-DNP), (dinitrophenyl)hydrazone of methoxyacetaldehyde (MAA-DNP).

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high toxicity of these compounds (*3*) precluded further biological studies.

During the course of these investigations, a striking difference in toxicity was noted between CMM and its corresponding carbethoxytriazene analog, 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene (CMC). This led to the hypothesis that these compounds might have very different fates in vivo. Studies were therefore initiated to determine the metabolic pathway of the (methylcarbamoyl)triazenes, focusing on 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene (CBzM) as a model compound. Results indicated that degradation of this compound was oxidative rather than hydrolytic and was most likely catalyzed by one or more isozymes of cytochrome P450. Similar results were obtained in preliminary studies of the metabolism of CMM. During the course of CBzM breakdown, two triazene metabolites were formed which were isolated and identified as 1-(2-chloroethyl)-3-benzyl-3-((hydroxymethyl)carbamoyl)triazene (CBzM-met1) and 1-(2-chloroethyl)-3-benzyl-3-carbamoyltriazene (CBzM-met2). A single triazene metabolite isolated from the incubation of CMM with microsomes was identified as 1-(2-chloroethyl)-3-methyl-3-((hydroxymethyl)carbamoyl)triazene (CMM-met1). These results suggested that the primary site of attack on these compounds was the methylcarbamoyl substituent, leading to its hydroxylation and, in the case of CBzM, its subsequent demethylation. In CBzM incubations, both the parent compound and the metabolites eventually completely disappeared, and the lack of appearance of new compounds bearing the triazene chromophore indicated that further processes led to the destruction of the triazene moiety. In the case of CMM, metabolism ceased with significant quantities of both the parent compound and triazene metabolite still present in incubation mixtures.

The above results indicated that the antitumor efficacy of the (methylcarbamoyl)triazenes may be attributable to a unique mechanism that does not involve hydrolytic deacylation or the formation of an alkanediazonium ion. However, the ultimate fate of these compounds remained unknown. In this report, we present evidence that oxidation of both CMM and CBzM also occurs at the 2-chloroethyl substituent. This oxidation leads to loss of the 2-chloroethyl group as chloroacetaldehyde. The resulting unstable triazene breaks down with loss of nitrogen to yield the corresponding urea.

Materials and Methods

Animals. Young adult male Fischer 344 rats (8−10 weeks) from the colony of the Frederick Cancer Research and Development Center were maintained on standard laboratory diet and given water *ad libitum*.

Chemicals. Triazenes were prepared, purified, and characterized according to the procedure of Smith *et al.* and were >99% pure as determined by NMR and UV/vis spectroscopy and high performance liquid chromatography (*1*, *2*). Triazene stock solutions were 100 or 300 mM in acetonitrile and were stored at −20 °C.

The (2,4-dinitrophenyl)hydrazone of chloroacetaldehyde (CAA-DNP) was prepared by dissolving 4.5 mmol of (2,4-dinitrophenyl)hydrazine in 4.5 mL of concentrated H_2SO_4, and diluting with 6.0 mL of water and 21.0 mL of 95% (v/v) ethanol. Chloroacetaldehyde (4.5 mmol) was then added as a 50% (w/v) aqueous solution. The resulting precipitate was collected by filtration and recrystallized from 95% (v/v) ethanol. ^{1}H NMR of the CAA-DNP derivative yielded the following: (CD_3CN, Me_4Si) δ 4.35 (2H, d, J = 5.7 Hz), 7.73 (1H, t, J = 5.7 Hz), 7.92 (1H, d, J = 9.6 Hz), 8.33 (1H, d of d, J = 2.6 Hz, J = 9.7 Hz), 8.96 (1H, d, J = 2.5 Hz), 11.04 (1H, broad). Electron impact mass spectrometry indicated an accurate mass of 258.0143 amu (calculated value of 258.0155 amu for molecular formula $C_8H_7N_4O_4Cl$). The compound decomposed above 152 °C.

The (dinitrophenyl)hydrazone of methoxyacetaldehyde (MAA-DNP) was prepared as described above for CAA-DNP, except that 4.5 mmol of methoxyacetaldehyde dimethyl acetal (neat) replaced the chloroacetaldehyde solution, and no recrystallization was performed. ^{1}H NMR of the MAA-DNP derivative yielded the following: (CD_3CN, Me_4Si) δ 3.38 (3H,s), 4.15 (2H, d, J = 5.0 Hz), 7.71 (1H, t, J = 5.0 Hz), 7.91 (1H, d, J = 9.6 Hz), 8.31 (1H, d of d, J = 2.6 Hz, J = 9.6 Hz), 8.96 (1H, d, J = 2.6 Hz), 11.00 (1H, broad). Electron impact mass spectrometry indicated an accurate mass of 254.0626 amu (calculated value of 254.0650 amu for molecular formula $C_9H_{10}N_4O_5$).

Benzylmethylurea was synthesized by slowly adding 25 mL of a solution of 0.5 M methyl isocyanate in methylene chloride to 25 mL of a solution of 0.6 M benzylamine in methylene chloride with constant stirring at 0 °C. When the addition was complete, the reaction mixture was stirred for an additional 2 h at room temperature. After extraction twice with 50 mL of 0.1 M HCl, the methylene chloride solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The resulting solid was recrystallized from an ethanol/water solution. ^{1}H NMR revealed the following: (CD_3CN, Me_4Si) δ 2.62 (3H, d, J = 4.8 Hz), 4.25 (2H, d, J = 6.2 Hz), 4.94 (1H, s), 5.45 (1H, s), 7.28 (5H, m). Electron impact mass spectrometry indicated an accurate mass of 164.0943 amu (calculated value of 164.0949 amu for molecular formula $C_9H_{12}N_2O$).

NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and benzylurea were purchased from Sigma Chemical Co. (St. Louis, MO). Methoxyacetaldehyde dimethyl acetal and chloroacetaldehyde were purchased from Aldrich (Milwaukee, WI).

Rat Liver Microsomes. Rat liver microsomes were prepared as previously described (*9*). The preparations contained approximately 40 mg/mL protein as determined by the method of Bradford (*10*).

Incubations of Triazenes with Rat Liver Microsomes. Incubation mixtures contained 0.1 M Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 1 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.2 unit/mL glucose 6-phosphate dehydrogenase, 3 mg of microsomal protein/mL, and the desired final concentration of triazene added as a stock solution in acetonitrile. In general, all components of the reaction mixture were combined except for the microsomes. Samples were warmed to 37 °C, and the microsomes were added to initiate the reaction. Aliquots were then removed at desired time points for asssay of triazene, benzylurea, benzylmethylurea, or chloroacetaldehyde concentration.

Assay of Triazene Metabolism. The concentrations of triazenes were determined by high performance liquid chromatography (HPLC) as described previously (*9*). In the case of CBzM, CMM was added to the samples as an internal standard. When CMM was to be assayed, the internal standard was 1-(2-chloroethyl)-3-methyl-3-acetyltriazene. The HPLC effluent was monitored by absorbance at 245 nm, and concentrations of the parent compounds were calculated from the area of the corresponding peak relative to the internal standard peak. The concentrations of CBzM-met1, CBzM-met2, and CMM-met1 were similarly estimated, based on the assumption that the molar absorption coefficients of these compounds would not differ significantly from those of their parent triazenes. Details of the eluting solvents and retention times of the compounds have been published (*9*).

Assay of Benzylurea and Benzylmethylurea Formation. Mixtures containing 300 μM initial concentrations of CBzM were incubated as described above. At desired time points, aliquots (100 μL) were combined on ice with 50 μL each of saturated barium hydroxide and 5% (w/v) zinc sulfate. The precipitates were removed by centrifugation, and the samples were subjected to HPLC on a column (4.6 × 250 mm) of Microsorb C18 (Rainin

Instrument Co.) eluted at a flow rate of 1 mL/min with methanol/water (30:70 v/v). The retention times for benzylurea and benzylmethylurea were 9.1 and 12.2 min, respectively. The effluent was monitored by absorbance at 250 nm. Quantitation of each urea was based on the area of the corresponding peak as compared to a standard curve prepared from the injection of samples containing 0.5−25 nmol of the authentic compounds. The response of the HPLC detector was linear with respect to urea quantity over this concentration range.

Assay of Chloroacetaldehyde Formation. Mixtures (3.5 mL total volume) containing an initial concentration of 300 μM CBzM or CMM were prepared as described above and incubated at 37 °C for 3 h. For quantitation standards, identical mixtures were prepared, except that the triazenes were replaced with solvent, and known concentrations (100−300 μM) of chloroacetaldehyde were added after 1.5 h of incubation. At the beginning and end of the incubation period, duplicate 100 μL samples were removed for assay of triazene and metabolite concentration. To the remaining (3 mL) reaction mixture was added 1.5 mL each of saturated barium hydroxide and 5% (w/v) zinc sulfate. After removal of the precipitates by centrifugation, the resulting supernatants were combined with 5.4 mL of water and 1.0 mL of a solution of 12.5 mM (2,4-dinitrophenyl)-hydrazine in 6 M HCl. Samples were mixed for 5 min at room temperature, at which time 5 mL of isooctane was added. After mixing for an additional 25 min, the isooctane layers were removed and extracted with 1 mL of acetonitrile. The acetonitrile extracts were evaporated to dryness, and the residues were dissolved in 100 μL of acetonitrile for HPLC analysis (*11*). Chromatography was performed on the column described above, eluted at 1 mL/min with a linear gradient beginning with methanol/water containing 5 mM ammonium acetate and 6 mM ammonium hydroxide (pH 9.0) (35:65 v/v) and ending with 100% methanol in a total volume of 30 mL. The column effluent was monitored for absorbance at 340 nm. The retention time for the peak corresponding to CAA-DNP was 19.4 min. Quantitation was based on the area of the peak corresponding to CAA-DNP obtained from triazene incubation mixtures relative to that of CAA-DNP quantitation standard mixtures. All incubations were performed in triplicate. The assay was linear with respect to chloroacetaldehyde concentations from 0 to 300 μM.

Isolation of Benzylmethylurea from CBzM Reaction Mixtures. A mixture (20 mL total volume) containing a 300 μM initial concentration of CBzM was incubated at 37 °C for 3 h. The reaction was terminated by the addition of 10 mL each of saturated barium hydroxide and 5% (w/v) zinc sulfate. The precipitate was removed by filtration, and the supernatant was extracted twice with 40 mL of methylene chloride. The methylene chloride solution was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 300 μL of methanol and purified by HPLC on the column described above eluted at 1 mL/min with methanol/water (40:60 v/v). The column effluent was monitored for absorbance at 250 nm, and the retention time for benzylmethylurea was 8 min. The partially purified samples from three such preparations were combined, concentrated, and subjected to a second purification by HPLC, in which the column was eluted with methanol/water (30:70 v/v). The purified material (retention time 12.2 min) was concentrated under reduced pressure and dissolved in deuterated acetonitrile for ^{1}H NMR and mass spectral analysis.

Isolation of MAA-DNP from CBzM Reaction Mixtures. A mixture (20 mL) was prepared, incubated, and treated with barium hydroxide and zinc sulfate as described for the isolation of benzylmethylurea. Following removal of the precipitate by filtration, the supernatant was combined with 7 mL of a solution of 12.5 M (2,4-dinitrophenyl)hydrazine in 6 M HCl. After mixing for 5 min at room temperature, the sample was overlaid with 35 mL of isooctane and mixed for an additional 25 min. The isooctane layer was then extracted twice with 7 mL of acetonitrile. The acetonitrile extracts from two such preparations were dried over sodium sulfate and evaporated to dryness. The residues were taken up in a total volume of 500 uL of acetonitrile for purification by HPLC. The column was eluted at 1 mL/min with a linear gradient beginning with methanol/water containing 5 mM ammonium acetate and 6 mM ammonium hydroxide, pH 9.0 (35:65 v/v), and ending with 100% methanol in a total volume of 60 mL. The MAA-DNP eluted with a retention time of 28.0 min. The purified material was evaporated to dryness under reduced pressure and dissolved in deuterated acetonitrile for ^{1}H NMR analysis.

Analyses. NMR spectra were obtained on a Varian VXRS 500 MHz spectrometer equipped with a SUN 4/110 data station.

Mass spectra were obtained on a VG 70−250 mass spectrometer (VG Analytical, Danvers, MA). The mass spectrometer was operated in the positive ion detection mode. Ion formation was by electron impact at a trap current of 100 μA and an electron energy of 70 eV. The ion source temperature was 180 °C. The instrument was scanned from m/z 650 to m/z 40 at 3 s/decade with a 0.5 s magnet reset time and a resolving power of 3000. The accelerating voltage was 6000 v. Accurate masses were obtained by peak matching using the mass spectrometer data system and perfluorokerosene as the source of reference peaks. Samples were introduced via the solid probe.

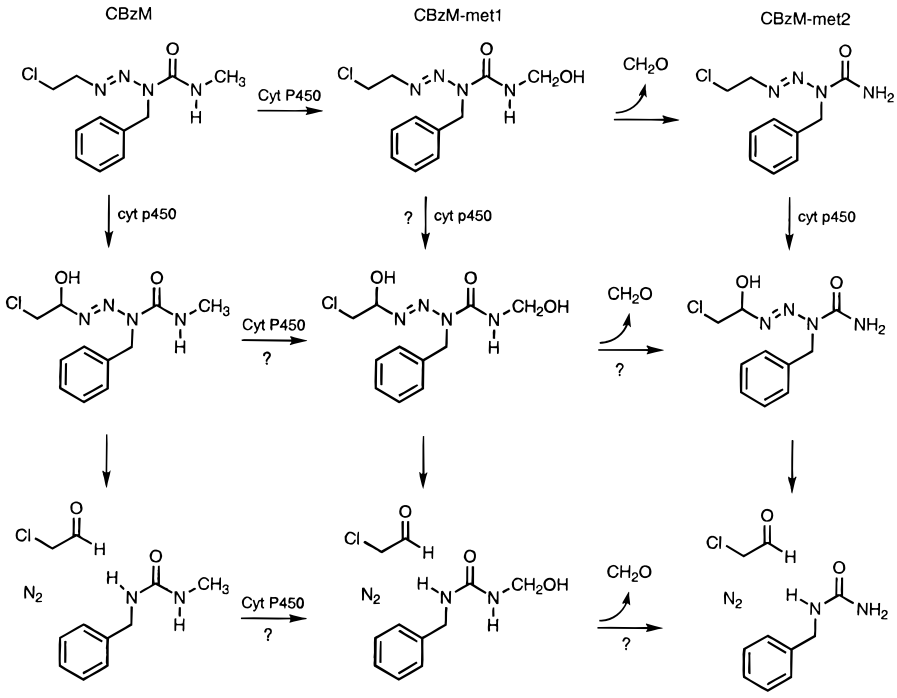
HPLC was performed on an instrument composed of a SCL-6B system controller driving two LC-6A pumps, an SPD6AV UV/vis spectrophotometric detector, and a CR601 Chromatopac data integrator, all obtained from Shimadzu Scientific Instruments.

Results

Formation of Benzylurea and Benzylmethylurea during CBzM Metabolism. Previously reported studies had shown that oxidation of the methylcarbamoyl substituent led to the formation of the ((hydroxymethyl)-carbamoyl)triazene and the carbamoyltriazene analogs of CBzM. However, it was also shown that these compounds were further metabolized oxidatively in a process that appeared to result in the destruction of the triazene moiety (*9*). Consideration of the structure of CBzM led to the hypothesis that a likely second site for metabolism might be the chloroethyl group. Hydroxylation of the carbon attached to N(1) of CBzM should result in the loss of the chloroethyl substituent as chloroacetaldehyde, and the breakdown of the triazene to yield nitrogen and benzylmethylurea. The same oxidation of the chloroethyl group of CBzM-met1 should produce (hydroxymethyl)benzylurea, whereas oxidation of CBzM-met2 should produce benzylurea (Scheme 1). Consequently, the elution times of commercially available benzylurea and synthetic benzylmethylurea on reverse-phase HPLC were ascertained in order that the presence of these potential metabolites could be investigated in CBzM reaction mixtures.

When incubation mixtures containing 300 μM CBzM, rat liver microsomes, and NADPH were incubated for 3 h at 37 °C, two new compounds were formed which coeluted on HPLC with benzylmethylurea and benzylurea. Coelution was tested by injecting the authentic compounds combined with the CBzM reaction mixtures. Single peaks were obtained for each compound in two separate solvent systems (methanol/water, 30:70 v/v, and acetonitrile/water, 20:80 v/v). Neither of these compounds was formed when microsomes, NADPH, or CBzM was omitted from the reaction mixtures, confirming that they were the products of oxidative metabolism of the triazene. Sufficient quantity of the compound that coeluted with benzylmethylurea was produced to allow isolation and purification from the reaction mixture. The ^{1}H NMR spectrum of the isolated metabolite was identical to that of synthetic benzylmethylurea (see Materials and Methods), and mass spectral analysis revealed the

Scheme 1. Proposed Pathway for the Oxidative Metabolism of CBzM



correct accurate mass of 164.0954 amu. It should be noted that a third compound having a retention time prior to both benzylurea and benzylmethylurea also appeared in CBzM reaction mixtures. This compound may have been (hydroxymethyl)benzylurea; however, the lack of authentic standard and the minute amount of this product precluded identification.

Figure 1 shows the time course of the metabolism of CBzM and the formation of CBzM-met1, CBzM-met2, benzylmethylurea, and the compound that coeluted with benzylurea. Benzylmethylurea, CBzM-met1, and CBzM-met2 come directly from the oxidation of CBzM. Therefore, these compounds appear immediately, as the concentration of CBzM decreases. In contrast, benzylurea should be formed from the oxidation of CBzM-met2. Consistent with this, we find that the peak coeluting with benzylurea does not appear until significant levels of CBzM-met2 have formed. It should be noted that the efficiency of oxidative metabolism of acyltriazenes is directly related to their lipophilicity (*9*). Because CBzM-met2 is more polar than CBzM, it should not compete effectively as a substrate for metabolism until its concentration reaches reasonable levels.

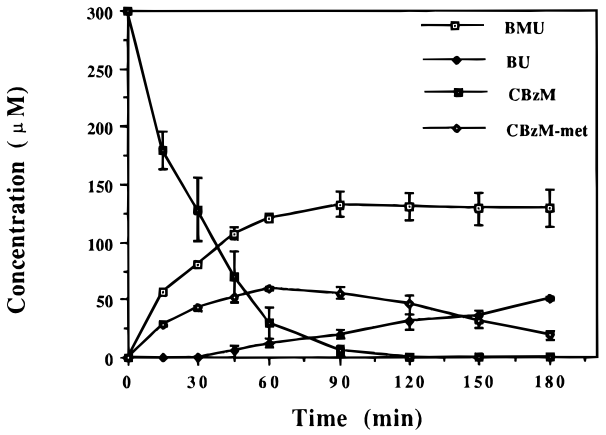


Figure 1. Time course for the metabolism of CBzM and the formation of CBzM metabolites. An incubation mixture containing an initial concentration of 300 μM CBzM was prepared as described in Materials and Methods. At the indicated times, duplicate samples were removed for assay of CBzM, CBzM-met1, and CBzM-met2 (CBzM-met, quantitated together), benzylmethylurea (BMU), and the compound coeluting with benzylurea (BU). Each data point represents the mean and standard deviation for three separate experiments.

Formation of Chloroacetaldehyde during the Metabolism of CBzM. If the metabolic pathway depicted in Scheme 1 is correct, then one would expect to detect chloroacetaldehyde in addition to the substituted ureas in CBzM reaction mixtures. In order to test this hypothesis, an assay was devised that could detect and quantitate chloroacetaldehyde as its (2,4-dinitrophenyl)-hydrazone derivative. Reaction mixtures containing 300 μM CBzM, microsomes, and NADPH were incubated for 3 h at 37 °C. Following reaction with (2,4-dinitrophenyl)-hydrazine, a compound was detected in these mixtures that coeluted with CAA-DNP synthetic standard on HPLC. This substance was not formed if CBzM, microsomes, or NADPH was omitted from the incubation.

In order to verify the identity of the putative CAA-DNP, the compound was purified from CBzM reaction mixtures. The ^{1}H NMR spectrum of the isolated material was similar, but not identical to that of synthetic CAA-DNP derivative (Materials and Methods). In particular, instead of a doublet at 4.35 with a coupling constant of 5.7 Hz as found in the CAA-DNP spectrum, the spectrum of the isolated metabolite showed a doublet at 4.15 with a coupling constant of 5.0 Hz. In addition, the metabolite spectrum showed that the coupling constant of the triplet at 7.73 was 5.0 Hz instead of 5.7 Hz as found in the CAA-DNP spectrum, and it contained a singlet corresponding to 3 protons at 3.38 not found in the spectrum of CAA-DNP. Furthermore, mass spectral analysis yielded a mass of 254.0638 amu for the metabolite, as opposed to 258.0155 amu expected for CAA-DNP. Clearly, this compound was not CAA-DNP.

Since chloroacetaldehyde was a highly likely metabolite from the breakdown of CBzM, we considered the possibility that CAA-DNP formed in the reaction mix-

tures was being altered during the purification procedure. To test this hypothesis, we subjected samples of synthetic CAA-DNP to the HPLC purification followed by NMR analysis. The results showed that the NMR spectrum of the recovered compound was identical to that of the material isolated from CBzM incubation mixtures, and not that of CAA-DNP. Furthermore, simply incubating CAA-DNP with solutions of methanol and water at concentrations used in the HPLC purification, followed by evaporation under reduced pressure, led to a similar change in NMR spectrum. These findings along with the spectral data suggested the hypothesis that the compound recovered from the HPLC was the (dinitrophenyl)-hydrazone of methoxyacetaldehyde (MAA-DNP), resulting from nucleophilic substitution of the chloro substituent by methanol. This was confirmed through the preparation of synthetic MAA-DNP standard, which coeluted exactly with CAA-DNP on HPLC and gave NMR and mass spectral data identical to those of the compound isolated from CBzM incubation mixtures.

We believe that the most likely explanation for the isolation of MAA-DNP from CBzM incubation mixtures is that CAA-DNP is converted to MAA-DNP either on the HPLC column or during the lengthy evaporation procedure after HPLC. The finding that this material is not formed in incubation mixtures in the absence of CBzM, microsomes, or NADPH indicates that it is the result of CBzM metabolism; however, there is no obvious reason to expect the direct formation of methoxyacetaldehyde from oxidative metabolism of CBzM. Since CAA-DNP is converted to MAA-DNP upon incubation with methanol/water mixtures, and since CBzM reaction mixtures are not exposed to methanol prior to HPLC injection, a conversion of CAA-DNP to MAA-DNP after the HPLC step is reasonable. Attempts to use different solvent systems and different purification techniques have not yet resulted in the isolation of CAA-DNP from CBzM reaction mixtures at an adequate level of purity to allow spectral confirmation. Nevertheless, we believe that the similarity in the behavior of the CBzM metabolite compared to that of the CAA-DNP synthetic standard provides strong evidence that the metabolite that we are measuring is chloroacetaldehyde.

Quantitation of CBzM Metabolites. A series of experiments were performed in order to quantitate CBzM and all metabolites formed during a 3 h incubation, beginning with a starting concentration of 300 μM CBzM. The results are summarized in Table 1. It is notable that, in this series of experiments, the same pattern of metabolites was obtained as was observed in the experiments shown in Figure 1, but the actual quantities of some of the metabolites differed. This variability was not uncommon with different preparations of microsomes.

Formation of Chloroacetaldehyde during the Metabolism of CMM. Initial studies of the metabolism of CMM had shown that, like CBzM, oxidation at the methylcarbamoyl substituent could occur, yielding CMM-met1 (*9*). However, it seemed quite likely that CMM should also be subject to metabolism at the chloroethyl substituent. Because the expected urea metabolites of CMM, 1,3-dimethylurea, methylurea, and 1-(hydroxymethyl)-3-methylurea are not easily detected on HPLC due to the absence of a chromophore, we searched instead for the presence of chloroacetaldehyde in CMM reaction mixtures. Results indicated that, when 300 μM CMM was incubated for 3 h at 37 °C with rat liver microsomes and NADPH, a compound was formed that coeluted with synthetic CAA-DNP (and MAA-DNP) on HPLC. This compound did not form if CMM, microsomes, or NADPH was omitted from the reaction mixtures. Table 2 summarizes results from a series of experiments in which the concentrations of CMM and its metabolites were quantitated in 3 h incubation mixtures. Consistent with earlier studies (*9*), CMM was not as extensively metabolized as CBzM under these conditions. Thus, high concentrations of CMM and CMM-met1 remained at the end of the incubation. However, significant levels of chloroacetaldehyde (58 μM) were detected.

Table 1. Metabolites Produced during the Breakdown of CBzM

	concentration (μM)[a]						
expt	CBzM[b]	CBzM-met1	CBzM-met2	BMU[c]	BU[d]	CAA[e]	total[f]
1	22.9 (3.7)	50.8 (1.6)	35.0 (3.2)	133 (4)	22.7 (4.1)	67.0 (5.8)	264
2	21.5 (4.7)	29.5 (5.8)	41.3 (4.4)	126 (7)	21.5 (8.3)	78.3 (1.3)	240
3	5.8 (1.1)	46.3 (16.8)	17.3 (4.6)	143 (24)	21.5 (8.3)	87.9 (3.0)	234
mean[g]	16.7 (9.5)	42.2 (11.2)	31.2 (12.4)	134 (8)	21.9 (0.7)	77.7 (10.5)	246

[a] Values represent the mean and (standard deviation) of triplicate incubation mixtures for each experiment. [b] Values represent remaining CBzM concentration at the end of the incubation. [c] Benzylmethylurea. [d] Benzylurea. [e] Chloroacetaldehyde. [f] Total of CBzM accounted for as the sum of remaining CBzM, CBzM-met1, CBzM-met2, benzylmethylurea, and benzylurea. [g] Mean and (standard deviation) of three separate experiments.

Table 2. Metabolites Produced during the Breakdown of CMM

	concentration (μM)[a]			
expt	CMM[b]	CMM-met1	CAA[c]	total[d]
1	214 (8)	45.5 (1.2)	32.3 (4.6)	292
2	199 (3)	42.8 (2.2)	34.0 (1.3)	276
3	226 (7)	49.5 (1.7)	49.3 (6.0)	324
4	117 (14)	91.2 (7.4)	116 (11)	324
mean[e]	189 (49)	57.2 (22.8)	57.9 (39.5)	304 (24)

[a] Values represent the mean and (standard deviation) of triplicate incubation mixtures for each experiment. [b] Values represent remaining CMM concentration at the end of the incubation. [c] Chloroacetaldehyde. [d] Total of CMM accounted for as the sum of remaining CMM, CMM-met1, and chloroacetaldehyde. [e] Mean and (standard deviation) of the four separate experiments.

Discussion

The results presented here indicate that the (methylcarbamoyl)triazenes CBzM and CMM are subject to oxidative metabolism at a minimum of two sites. These sites are the methylcarbamoyl and the chloroethyl substituents. Hydroxylation of the methylcarbamoyl group may lead to demethylation, at least in the case of CBzM, but does not directly result in breakdown of the triazene or the generation of an obvious alkylating agent (*9*). In contrast, hydroxylation of the chloroethyl group results in destruction of the triazene moiety, with formation of chloroacetaldehyde, a substituted urea, and presumably molecular nitrogen. The oxidation of the chloroethyl group of CBzM (and its methylcarbamoyl metabolites) is supported by the following evidence. Chloroacetalde-

hyde has been identified as its (2,4-dinitrophenyl)hydrazone in CBzM reaction mixtures by its coelution with synthetic CAA-DNP standard on HPLC, and by its quantitative conversion to MAA-DNP under conditions of analysis, as also observed for the CAA-DNP standard. The expected urea metabolite of CBzM, benzylmethylurea, has been purified and positively identified by comparison of its ^{1}H NMR and mass spectrum with those of authentic compound. The urea expected from CBzM-met2, benzylurea, has been tentatively identified on the basis of coelution on HPLC with standard. An assay for the urea expected from CBzM-met1, (hydroxymethyl)-benzylurea, is not yet available. However, it is unlikely that large quantitities of this compound are formed, as we observe only a very minor peak that demonstrates the expected HPLC behavior. It should be noted that formation of (hydroxymethyl)benzylurea from CBzM-met1 might be followed by demethylation, in which case it would be converted to and quantitated with benzylurea.

In the experiments summarized in Table 1, 287 μM out of the 300 μM CBzM was actually metabolized over the 3 h incubation. We were able to account for 81% of the metabolized compound as follows: CBzM-met1 and CBzM-met2 (26%), benzylmethylurea (47%), and benzylurea (8%). It is interesting to note that, if the pathway in Scheme 1 is correct, the formation of benzylmethylurea (47%) represents oxidation of CBzM first at the chloroethyl group, whereas the formation of the other metabolites combined (34%) represents oxidation first at the methylcarbamoyl group. The preponderance of benzylmethylurea suggests that oxidation of CBzM at the chloroethyl group is favored over oxidation at the methylcarbamoyl group. It is not known whether the 19% of the triazene that has not been accounted for represents experimental error, or whether it indicates that some of the parent compound decomposes by an as yet unknown pathway. Alternatively, it is possible that the present assays simply do not detect all of the metabolites formed, including (hydroxymethyl)benzylurea. In addition, the ureas themselves may be subject to still further metabolism such as aromatic or benzylic hydroxylation, although the stability in the level of benzylmethylurea from 90 to 180 min of incubation (Figure 1) does not support this.

If the suggested pathway in Scheme 1 is correct for CBzM oxidation, then one would expect to detect chloroacetaldehyde in a concentration equal to the sum of the concentrations of the substituted urea metabolites. This was not the case, as we detected approximately 156 μM total urea, but only 77.7 μM chloroacetaldehyde in the experiments shown in Table 1. This discrepancy may be simply the result of error compounded from multiple assays, but it also raises two critical questions. First, we must consider the possibility that a portion of the urea metabolites is formed by simple chemical breakdown involving heterolysis of the N(2)−N(3) bond, which is known to occur at neutral pH, and which would result in the formation of the 2-chloroethanediazonium ion (*4*). However, as mentioned previously, the rate of this reaction is very slow relative to our 3 h incubation period, and the fact that we do not detect any of the urea metabolites in the absence of NADPH or microsomes suggests that enzymatic oxidation is required for their formation under our conditions. The second important question that we must consider is the possibility that not all chloroacetaldehyde formed during the incubation is detected in our assay. This is a distinct possibility, as indicated by our finding that, when a known quantity of chloroacetaldehyde was added to incubation mixtures and subjected to the derivatization and extraction procedure, only 20% of it was recovered as CAA-DNP on HPLC analysis. The low recovery could have been due to chemical reaction of the chloroacetaldehyde with reaction mixture components, to metabolism of chloroacetaldehyde to chloroacetic acid (*12*), and/or to partial losses during sample processing. In order to compensate for this problem, we attempted to simulate the environment of endogenously generated chloroacetaldehyde in our quantitation standards by adding known concentrations of the compound to microsome incubation mixtures at the midpoint of the incubation period (see Materials and Methods). It is not known, however, whether this technique truly produces standards that accurately reflect the fate of the endogenously generated molecule. Therefore, the exact quantities of chloroacetaldehyde produced during the metabolism of CBzM cannot be certain. Interestingly, in the CMM metabolism studies, the measured quantity of chloroacetaldehyde accounted fully for the quantity of CMM that was degraded, but not accounted for as CMM-met1 (Table 2).

In spite of the difficulties with chloroacetaldehyde quantitation, this compound is clearly produced from the chloroethyl substituent of CBzM and CMM, whereas we have found no evidence for the formation of the 2-chloroethanediazonium ion during metabolic breakdown. These findings are consistent with preliminary in vivo studies which failed to show significant levels of chloroethylation or methylation of DNA in the livers of rats treated with single doses of either CBzM or CMM, when CMC caused measurable DNA alkylation under the same conditions. Threrefore, it now appears unlikely that the formation of an alkanediazonium ion can account for the documented antitumor efficacy of CMM.

The critical question arising from these results is whether chloroacetaldehyde formation during CMM metabolism respresents a simple mechanism for breakdown and excretion of the triazene, or an alternative explanation for CMM's antitumor effects. Chloroacetaldehyde is a known metabolite of both vinyl chloride, an important industrial carcinogen, and isophosphamide, a clinically useful cancer chemotherapeutic agent (*13*−*16*). However, the exact role of CAA, if any, in the bological effects of these compounds remains uncertain. CAA is a known mutagen presumably due to its reaction primarily with adenine and cytosine bases of DNA to form 1,N^6-ethenoadenine and 3,N^4-ethenocytosine, respectively (*17*−*22*). DNA cross-linking by chloroacetaldehyde has also been reported (*23*). However, the kinetics of the reaction of CAA with DNA are slow compared to the kinetics of its reaction with protein (*16*).

Although further work will be necessary to determine the exact mechanism for the antitumor action of CMM, the fact remains that CMM has demonstrated a remarkably low degree of toxicity relative to its efficacy. This is in marked contrast to CMC, which breaks down to yield a highly reactive alkanediazonium ion in vivo, and which is highly toxic. Thus, one may ask whether CMM may represent a prototype for a new class of alkylating agents in which the ultimate active species is of lower reactivity than previously thought necessary for antitumor activity, and from which a major benefit of reduced toxicity may be achieved.

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