

Microsomal Metabolism of Triazenylimidazoles¹

Donald L. Hill

Keating-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

SUMMARY

The antitumor agents 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide (DIC) and 5-[3,3-bis(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide (BIC) are substrates for NADPH-requiring microsomal enzymes of mouse liver. The products of DIC oxidation are 5-aminoimidazole-4-carboxamide (AIC) and formaldehyde. Those for BIC are AIC and, presumably, 2-chloroacetaldehyde. For DIC, the reaction has a pH optimum of 9.0; and the Michaelis constant (K_m) is 0.25 mM. At lower pH values, the K_m is not greatly increased; but there is a sharp rise in the K_m values above pH 9.0. For the enzyme-catalyzed production of AIC from BIC, the pH optimum is 7.5; the K_m value for BIC is 0.47 mM.

Of a variety of tissues tested for enzymatic activity, only liver accomplishes the conversion of DIC and BIC to AIC. Most of the activity in the liver is located in the microsomal fraction, although detectable activity is present in washed mitochondria. For liver microsomes, the rate of reaction for BIC is greater than that for DIC, but apparently neither rate is fast enough to allow extensive metabolism of large doses of these agents.

INTRODUCTION

Several triazenylimidazoles have activity against experimental animal tumors (1, 24, 32), and 2 of these, DIC² and BIC (Chart 1), have been evaluated in patients (2, 4, 6, 11, 15, 17). DIC has consistent activity against malignant melanoma. With single-agent therapy, 20% of the patients respond.

Activation of DIC is required to produce potent biological activity. One type of activation is photochemical, forming 5-diazoimidazole-4-carboxamide, which can inhibit the growth of bacteria (19, 21, 22). Light-activated DIC is also toxic to cultured mammalian cells (7). An enzymatic, activating reaction involves *N*-demethylation of DIC, giving rise to formaldehyde and, presumably, the monomethyl

compound, which is relatively unstable and decomposes to liberate N₂, AIC, and a reactive methyl carbonium ion (10, 24, 25, 29). 7-Methylguanine can be detected in nucleic acids present in *in vitro* systems accomplishing the enzymatic activation of DIC (16), and in the urine and nucleic acids of rats given DIC (30). A similar mechanism of activation applies for 1-phenyl-3,3-dialkyltriazenes (20).

The markedly greater activity of BIC, compared with that of DIC, against L1210 leukemia (24) and the presence of the nor-nitrogen mustard group might suggest that this agent is activated by a mechanism different from other dialkyltriazenes, perhaps in a reaction involving dissociation to nor-nitrogen mustard (31). However, for DIC, 1-phenyl-3,3-dimethyltriazene, and BIC, the proposed intermediate monoalkyltriazenes have been prepared and have been found to possess anticancer activity (23, 24, 25, 28), indicating that microsomal oxidation may also be involved in the activation of BIC.

This report confirms that, in addition of DIC, BIC is a substrate for a microsomal enzyme. Also, the optimal conditions and kinetic properties are established for enzymes utilizing DIC and BIC as substrates.

MATERIALS AND METHODS

[2-¹⁴C]DIC (15.5 μ Ci/ μ mole) and [2-¹⁴C]BIC (34.4 μ Ci/ μ mole) were prepared by Monsanto Research Corp., Dayton, Ohio, and were obtained from Dr. Robert Engle, Drug Development Branch, National Cancer Institute, Bethesda, Md. [2-¹⁴C]AIC was purchased from Calatomic, Los Angeles, Calif. Except for the transformation product of BIC (26), which forms rapidly in solution and on paper chromatography, no detectable radioactive impurities were evident, and the compounds were used without dilution with nonradioactive material. 2-Chloroacetaldehyde was purchased from K and K Laboratories, Inc., Plainview, N. Y.

During this investigation, the stability of DIC and BIC was a major concern. To avoid the light-catalyzed decomposition of DIC (27), 1-ml samples of DIC in 0.05 N HCl were stored frozen in the dark. No detectable radioactive impurities appeared within 4 months. In addition to light-catalyzed dissociation, BIC in solution undergoes an internal alkylation to its ionic transformation product (26). This chemical reaction effectively limited the time for which the enzymatic reaction could take place. The formation of AIC from BIC was linear with time for only 4 min, the time used for all assays of BIC metabolism.

Animals used in these experiments were female DBA/2 mice, 2 months old and weighing 18 to 23 g. Sarcoma 180

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² The abbreviations used are: DIC, 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide; BIC, 5-[3,3-bis(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide; and AIC, 5-aminoimidazole-4-carboxamide; LD₁₀, the amount of agent killing 10% of the animals in a toxicity test.

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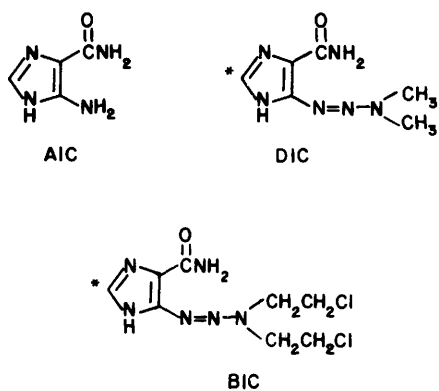


Chart 1. Structural formulas of AIC, DIC, and BIC; * position of the ^{14}C label.

tumors, 8 days after passage, were excised from the subaxillary region of female Swiss mice. For preparation of subcellular fractions, tissues from 3 or more mice were excised, cooled, and homogenized together in 3 volumes of 0.25 M sucrose. The preparations were centrifuged at $800 \times g$ for 5 min. The supernatant was removed and centrifuged at $9,000 \times g$ for 15 min to obtain the mitochondrial fraction. Microsomes were prepared from the resulting supernatant by centrifuging at $100,000 \times g$ for 45 min. Both mitochondria and microsomes were resuspended and washed by centrifugation in 0.25 M sucrose. Protein was determined by the method of Lowry *et al.* (14).

The standard assay for [^{14}C]DIC metabolism contained the following: [^{14}C]DIC, 35 nmoles, dissolved in 50 mM HCl; an equivalent amount of 50 mM NaOH; 10 μmoles of sodium glycinate buffer (pH 9.0 at 37°); 1 μmole of TPNH; liver microsomes equivalent to 30 mg of liver; and water in a total volume of 230 μl . Incubation was for 15 min at 37° . The assay for [^{14}C]BIC metabolism was the same, except that [^{14}C]BIC, 75 nmoles, was substituted for [^{14}C]DIC, and Tris buffer (pH 7.5 at 37°) replaced the sodium glycinate. These assays were allowed to proceed for 4 min at 37° .

One colorimetric test involved the detection of AIC after its diazotization and coupling with *N*-(1-naphthyl)ethylenediamine (3). The colored complex was measured at 540 nm. Another colorimetric test detected formaldehyde after its reaction with acetylacetone (5). The colored complex formed by this procedure was measured at 415 nm. For these tests, the standard reaction system was scaled up 2-fold, and the amount of nonradioactive DIC and BIC added was 250 nmoles. (Smaller amounts were used in the radioactivity assays to conserve material and to allow a higher percentage of conversion.) All manipulations involving DIC and BIC were performed under subdued light, and NADPH was omitted from controls.

Reactions were started by immediately adding a freshly prepared solution of the triazenyylimidazole and were stopped by adding 50 μl of ethanol. Portions (50 μl) were streaked onto paper strips, where substrates and metabolites were separated by paper chromatography. For separation of AIC and DIC, *n*-butyl alcohol:ethanol:water (4:1:1, by volume) was used. For BIC, the samples were allowed to

stand for 1 hr prior to streaking, at which time all of the unmetabolized BIC was present as the transformation product, 1-(2-chloroethyl)-3-(5-carbamoylimidazole-4-yl)- Δ^2 -1,2,3-triazolinium chloride. This compound was separated from AIC in a solvent of isopropyl alcohol:15 N ammonium hydroxide:water (80:5:15, by volume). The strips were scanned with a radiochromatogram scanner, and the amount of enzymatic product was calculated. For the colorimetric assays, reactions were stopped by adding 0.3 ml of 0.6 N perchloric acid, and the appropriate amount of NADPH was added to controls at this time. Known amounts of AIC or formaldehyde were added to some tubes to provide internal standards. To determine the tissue distribution of enzymes, standard assay conditions were used, except that 15 mg of homogenized tissue replaced microsomes in each assay.

All reactions were performed in duplicate and were linear with time over the period of incubation. The amounts of enzyme and NADPH were optimal (data not presented).

RESULTS

In addition to characterizing AIC as a diazotizable amine formed in the microsomal assay systems, this compound was further identified as a metabolite of both DIC and BIC by cochromatography with authentic [^{14}C]AIC on paper strips in 5 different solvents differing widely in composition and in R_F values for AIC (results not shown). When radioactivity and colorimetric assays were performed on the same microsomal systems, comparable results were obtained. In the *n*-butyl alcohol:ethyl alcohol:water chromatographic system, AIC had an R_F value of 0.35 and DIC had a value of 0.54. In the isopropyl alcohol:ammonia:water system, the R_F for AIC was 0.40 and that for the transformation product of BIC was 0.26.

The relationships between reaction rates and pH for both DIC and BIC are presented in Chart 2. For DIC, the reaction rate rises sharply between pH 6.5 and 8.8. Above

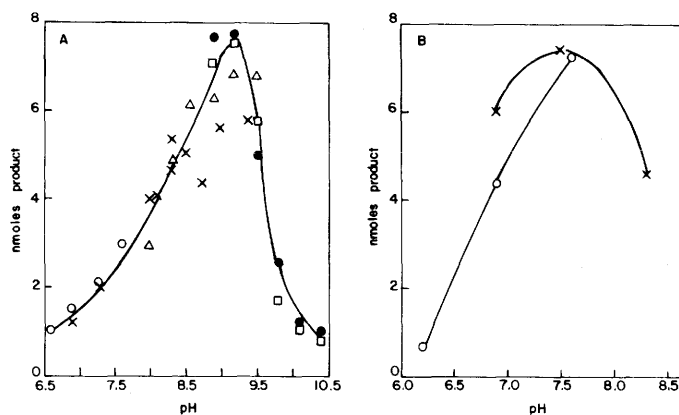


Chart 2. Effect of pH on DIC and BIC metabolism by liver microsomes. *A*, DIC metabolism, as measured in the radioactivity assay; *B*, BIC metabolism, as measured in the colorimetric assay; O, potassium phosphate buffer; X, Tris-chloride buffer; Δ , sodium barbital buffer; \square , sodium glycinate buffer; \bullet , sodium borate buffer. Equivalent concentrations of each buffer were used.

pH 9.2, it falls precipitously. Sodium glycinate, sodium borate, and sodium barbital were essentially equivalent in allowing the maximum rate of reaction. For BIC, the reaction rate reached a maximum at pH 7.5.

For both DIC and BIC metabolism, neither NAD nor NADP allowed a reaction rate greater than 10% of that for NADPH. However, the reaction rate with NADH and DIC was 20% of that for NADPH; and the reaction rate for NADH and BIC was 12% of that with NADPH. Magnesium chloride (5 mM) had no effect on either reaction.

When colorimetric assays for both AIC and formaldehyde were performed simultaneously on 4 identical reaction systems containing DIC as a substrate, 7.2 ± 0.6 nmoles (S.D.) of AIC and 9.8 ± 2.0 nmoles of formaldehyde were formed. 2-Chloroacetaldehyde, a putative product of BIC, gave no color in the test for formaldehyde and, in an assay of BIC metabolism where 3.6 nmoles of AIC were produced, less than 0.3 nmole of formaldehyde equivalents was present.

Radioactivity assays were used to determine the kinetics for microsomal metabolism of DIC (Chart 3). At pH 8.9, the K_m for DIC is 0.25 ± 0.02 mM (S.D. for 3 separate determinations). This value increases to 1.33 mM at pH 9.8. The V_{max} , which is 29 pmoles/min/mg liver tissue, does not change over this pH range. In a separate experiment with sodium barbital buffer at constant ionic strength, the K_m value for DIC increased from 0.25 to 0.40 mM as the pH decreased from 8.5 to 7.5. Again, there was no change in the V_{max} value (results not shown). For microsomal metabolism of BIC, a K_m value of 0.47 ± 0.06 mM (S.D. for 3 separate determinations) was derived (Chart 4). The V_{max} value was 83 pmoles/min/mg tissue.

Although BIC was a potent inhibitor of the microsomal metabolism of DIC, the reverse was not true (Table 1). With

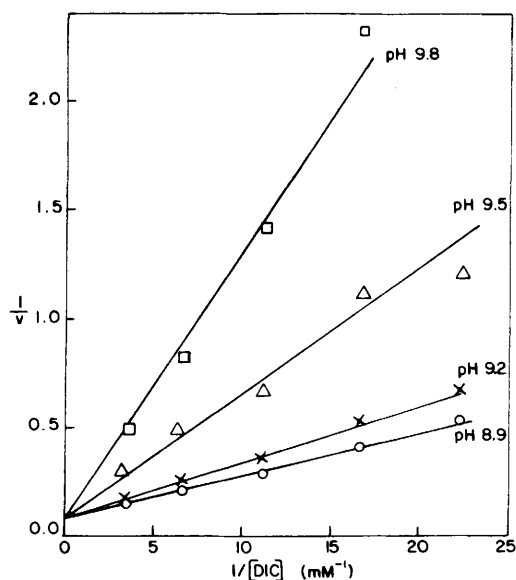


Chart 3. Lineweaver-Burk plots for microsomal metabolism of DIC at different pH values. Sodium glycinate was present as buffer, and the standard radioactivity assay was used. Values for the *ordinate* are reciprocals of nmoles of AIC formed.

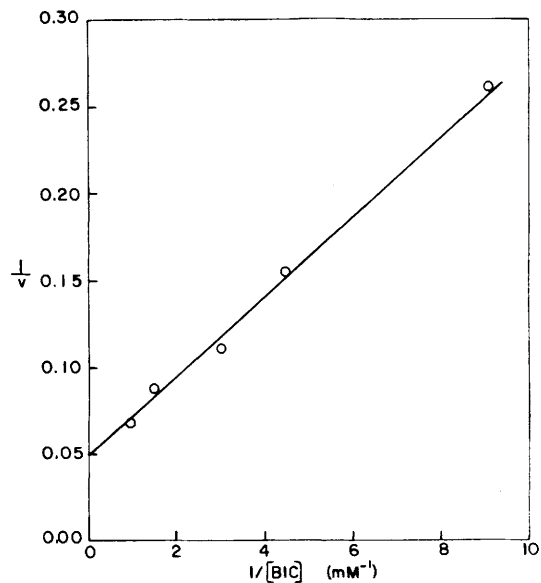


Chart 4. Lineweaver-Burk plot for microsomal metabolism of BIC. The standard colorimetric assay was used. Values for the *ordinate* are reciprocals of the nmoles of AIC formed.

Table 1
DIC and BIC as reciprocal inhibitors of their microsomal metabolism

Substrate	Inhibitor (mM)	Product (nmoles)	Relative activity
$[^{14}\text{C}]\text{BIC}$ (0.33 mM)	None	3.5	100
	DIC (0.44)	3.5	100
	DIC (0.66)	3.5	100
	DIC (1.10)	3.2	91
$[^{14}\text{C}]\text{DIC}^a$ (0.15 mM)	None	1.7	100
	BIC (0.11)	0.6	35
	BIC (0.22)	0.4	24
	BIC (0.44)	0.2	12

^a For this assay, the time of incubation was shortened to 4 min.

an inhibitor:substrate ratio of 3:1, BIC inhibited the formation of AIC from DIC by 88%. With a similar inhibitor:substrate ratio for the reciprocal test, DIC inhibited BIC metabolism only 9%.

In both radioactivity and colorimetric tests of homogenates of various mouse tissues and serum, enzymatic activity for DIC was found only in liver. Neither kidney, spleen, brain, muscle, intestine, lung, serum, nor Sarcoma 180 tumor tissue had a detectable amount of activity. Considered on the basis of equivalent amounts of protein, the maximum that could be present in any of these tissues is 10% of that in the liver. To determine the subcellular distribution of enzymes in liver tissue, homogenized liver, washed mitochondria, washed microsomes, and $100,000 \times g$ supernatant were tested in the colorimetric assay. The mitochondrial fraction contained 21% of the total cellular activity, microsomes contained 79%, and the supernatant had no detectable activity. Similar preparations from Sarcoma 180 tissue showed no detectable activity in any fraction.

DISCUSSION

This report confirms the proposed microsomal metabolism of BIC (28). The immediate product of this metabolism is very likely a hydroxylated BIC that quickly converts to the monoalkyl derivative and highly reactive 2-chloroacetaldehyde. The monoalkyl derivative is chemically unstable (28), decomposing to AIC and, presumably, N_2 and a chloroethyl carbonium ion. This report also confirms that, in the microsomal metabolism of DIC, more equivalents of formaldehyde than AIC are produced (29). The basis for this is not known.

The K_m for DIC varies with pH of the incubation system, rising sharply above pH 9.0, but the V_{max} does not change in the pH range of 7.2 to 9.8. Since titration curves for DIC reveal no pK_a value in this pH range (R. James and D. L. Hill, unpublished results), a likely explanation for this is that the enzyme exists in equilibrium with inactive ionic forms at low and high pH values. If so, an increase in the amount of substrate would allow more of the active form of the enzyme to be bound as the enzyme:substrate complex. Removal of the free, active enzyme would result in more of the inactive enzyme being converted to the active form. At infinitely high substrate concentrations, all of the enzyme would exist as the enzyme:substrate complex.

Apparently, neither the colorimetric nor the radioactivity assay is sensitive enough to detect the small amount of enzymatic activity previously observed in Sarcoma 180 cells (16).

The basis for the lack of reciprocal inhibition by BIC and DIC is not clear, but it must involve something more than simple competitive inhibition. For microsomal enzymes of mouse liver, we have previously observed lack of reciprocal inhibition by cyclophosphamide and nicotine (9) and by cyclophosphamide and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (8).

Although both DIC and BIC are metabolized by microsomal enzymes at appreciable rates, a large proportion of the administered drugs may not be acted upon enzymatically in the time that they remain in the body of the mouse. From Charts 3 and 4, V_{max} values for DIC and BIC are 29 and 83 pmoles/min/mg liver tissue, respectively. Assuming that 1 g of liver is present and that half-maximal conditions for metabolism exist continuously in this tissue, a mouse could metabolize 15 and 42 nmoles of DIC and BIC, respectively, in 1 min. The single-dose LD_{10} values for these compounds are, respectively, 68 and 25 μ moles/20-g mouse (F. M. Schabel, unpublished results). Accordingly, a mouse would require 75.5 hr to metabolize an LD_{10} dose of DIC and 9.9 hr for an LD_{10} dose of BIC. A slow rate of metabolism of DIC is probably related to the fact that large amounts of unchanged DIC are excreted by humans (13) and by dogs (10). The chemical instability of BIC (26) precludes metabolism of this agent over such a long period of time. In contrast, chemotherapeutic nitrosoureas are rapidly metabolized by liver microsomes, and very little of the intact drugs is excreted (8, 12, 18).

There are, of course, hazards in extrapolating from assays of microsomal enzymes to metabolism in intact animals.

However, such assays can serve to reinforce pharmacological studies of drug metabolism, and a trend toward rapid or slow metabolism can be established.

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