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IRREVERSIBLE INHIBITION OF THE CYTOSOLIC METABOLISM OF *N*-HYDROXY-2-ACETYLAMINOFLUORENE BY ITS GLYCOLYL ANALOG

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SUMMARY

The glycolyl hydroxamic acid derivative of 2-aminofluorene was found to be a potent inhibitor of its own metabolism and the metabolism of *N*-hydroxy-2-acetylaminofluorene by rat liver cytosol. The inhibition was irreversible, as well as time and concentration dependent, which indicates a suicide-inhibition type of metabolism. There was a direct correlation between the inhibition of *N*-hydroxy-2-acetylaminofluorene disappearance and 2-acetylaminofluorene formation. In contrast, both the glycolyl and acetyl hydroxamic acid derivatives were metabolized to a similar extent by enzymes in the microsomal fraction.

INTRODUCTION

The genotoxicity of 2-acetylaminofluorene (AAF) in rats requires the initial metabolic activation by microsomal enzymes to give *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) as a proximate metabolite. Further activation of this hydroxamic acid by cytosolic enzymes such as *N,O*-acyltransferase and sulfotransferase results in the formation of ultimate carcinogens and mutagens [6,11,15]. One important consideration of the bioactivation of hydroxamic acids by such enzymes is the ability of some of these compounds to inhibit the enzymes. Certain hydroxamic acids, especially N-OH-AAF, have been shown to be suicide substrates for *N,O*-acyltransferase [2,8,12,13]. In our studies to determine the metabolic fate of 2-aminofluorene-

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Abbreviations: AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; HPLC, high pressure liquid chromatography; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; N-OH-GAF, *N*-hydroxy-2-glycolylaminofluorene.

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derived hydroxamic acids, we found that the glycolyl derivative, *N*-hydroxy-2-glycolylaminofluorene (N-OH-GAF), was a potent inhibitor of N-OH-AAF metabolism by rat liver cytosolic enzymes.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. N-OH-AAF and N-OH-GAF were synthesized from *N*-hydroxy-2-aminofluorene by methods reported elsewhere (Corbett et al., submitted, 'Mutagenesis'). Dialysis tubing (Spectrapor m.w. cutoff 12,000–14,000) was obtained from Fisher Scientific Co.

Rats (Sprague–Dawley, male, 100–150 g) were sacrificed by cervical dislocation after halothane anesthesia. The livers were removed, perfused with ice-cold 0.9% NaCl, and then homogenized in three volumes (w/v) of 20 mM Tris –1.15% KCl buffer (pH 7.4). The homogenate was centrifuged at 9000 × *g* for 20 min, then the post-mitochondrial supernatant was centrifuged at 100,000 × *g* for 60 min. The microsomal pellet was resuspended in Tris–KCl buffer. Protein concentrations of the 100,000 × *g* supernatant (cytosolic) and microsomal fractions were determined by the Bradford method [3] using bovine serum albumin as standard.

The reaction mixture (5 ml) contained either ethanol (2.5–10 μl) as control, or 2.5–10 μl of a 10 mM solution of either N-OH-AAF or N-OH-GAF in ethanol, and 2 or 4 mg of microsomal or cytosolic protein per ml of Tris–KCl buffer, and was incubated for a specified time at 37 °C. The microsomal incubations also contained a NADPH generating system consisting of 0.5 mM NADP, 7.5 mM $MgCl_2 \cdot 6H_2O$, 7.5 mM glucose-6-phosphate, and 0.05 unit of glucose-6-phosphate dehydrogenase per ml of reaction mixture. Each reaction was stopped by the addition of an equal volume of ice-cold methanol. After centrifugation to remove the protein precipitate, the supernatant was analyzed to determine substrate disappearance and AAF formation (in the case of N-OH-2-AAF as substrate). An aliquot of the methanol supernatant was analyzed with a Waters high pressure liquid chromatography (HPLC) system using a reverse phase C18 μBondapak column. The samples were eluted off the column with a solvent system of 0.01 M KH_2PO_4/H_3PO_4 buffer (pH 3.5), which contained 0.01% desferoxamine methanesulfonate (Desferal Mesylate) in 55% methanol at a flow rate of 1.5 ml/min. The eluate was monitored at 280 nm with a Waters Model 440 Detector. The retention times of N-OH-AAF, N-OH-GAF and AAF were 7.6, 6.0 and 9.6 min, respectively.

RESULTS

As shown in Fig. 1, N-OH-AAF was rapidly metabolized by cytosolic enzymes. By 120 min, over 95% of the added substrate was depleted from the incubation. Concomitant with the disappearance of N-OH-AAF was the appearance of AAF (Fig. 1). AAF was identified as a metabolite by comparison

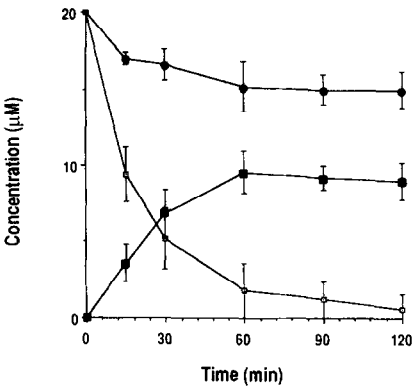

Fig. 1. Time course of the metabolism of N-OH-AAF and N-OH-GAF by cytosolic enzymes. N-OH-AAF or N-OH-GAF (20 μM) was incubated with 2 mg/ml of cytosolic proteins. Aliquots of 0.5 ml were removed at 0–120 min and analyzed by HPLC methods. Metabolism was determined by substrate disappearance (□, N-OH-AAF; ●, N-OH-GAF) and AAF formation (■). Values plotted are mean ± S.D. from 4–5 experiments.

of HPLC retention times and UV spectra with those of an authentic standard. The amount of AAF formed at 2 h was 9 μM, which was 45% of the original amount of N-OH-AAF (20 μM). The identities and amounts of other metabolites were not determined. On the other hand, since only 25% of the initially added N-OH-GAF was consumed within 60 min, and no further decrease in substrate was seen for up to 2 h, it appeared that N-OH-GAF inhibited its own metabolism (Fig. 1). Under the experimental conditions stated in Fig. 1, greater than 120 μM of N-OH-AAF was required for the inhibition of its own metabolism to a similar degree as that observed with 20 μM N-OH-GAF (data not shown).

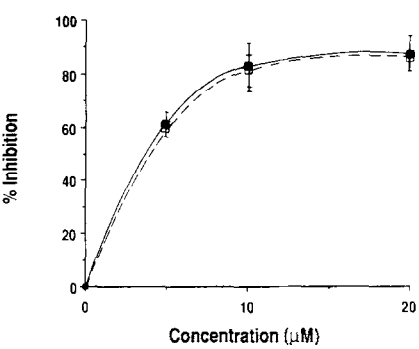

Fig. 2. Effect of N-OH-GAF concentration on the inhibition of N-OH-AAF metabolism. Cytosolic fraction (2 mg/ml) was preincubated with N-OH-GAF (0–20 μM) for 60 min, then N-OH-AAF (20 μM) was added and its disappearance (□) as well as AAF formation (■) after 30 min were determined by HPLC methods. Plotted are mean ± S.E.M. from 3 experiments. Values are expressed as % inhibition relative to controls without N-OH-GAF pretreatment.

TABLE 1

THE IRREVERSIBLE INHIBITION OF N-OH-AAF METABOLISM BY N-OH-GAF

Treatment	Preincubation	Dialysis	% N-OH-AAF[d] metabolized
(1) Control[a]	+	–	88 ± 3
N-OH-GAF	+	–	13 ± 9
(2) Control[b]	+	+	77 ± 6
N-OH-GAF	+	+	25 ± 9
(3) Control[c]	–	–	89 ± 1
N-OH-GAF	–	–	76 ± 7

[a]Cytosolic fraction (4 mg/ml) was preincubated with ethanol (control) or N-OH-GAF (20 μM) for 60 min, then N-OH-AAF (20 μM) was added and the incubation was continued for 30 min.

[b]Same as in (a) except that after the preincubation, aliquots were dialyzed for 5 h before N-OH-AAF addition.

[c]Ethanol or N-OH-GAF was added at the same time as N-OH-AAF to the cytosolic preparation, then the incubation was continued for 30 min.

[d]The % N-OH-AAF metabolized was determined by the amount of N-OH-AAF metabolized after 30 min divided by the amount initially added. Values are mean ± S.E.M. from 2–3 experiments.

To determine the nature of N-OH-GAF inactivation of cytosolic enzymes, we examined whether the inactivation reaction was time and concentration dependent. The cytosolic fraction was preincubated with N-OH-GAF (0–20μM) for various time periods (0–60 min), then N-OH-AAF was added and the rate of N-OH-AAF metabolism was determined. In these experiments, the maximum concentration of N-OH-GAF used for preincubation was 20 μM, while a higher concentration of N-OH-AAF (100 μM) was used for subsequent incubation, so that inhibition due to competition for active sites would be minimized. Results from these studies showed that N-OH-GAF inhibited both the disappearance of N-OH-AAF and the formation of AAF. This inhibition required a preincubation period of 30 min with 20 μM N-OH-GAF in order to cause more than 80% inactivation of the enzyme activity for N-OH-AAF metabolism (data not shown). The inhibition of N-OH-AAF metabolism by N-OH-GAF was also found to be concentration dependent (Fig. 2). Both the extent of inhibition of N-OH-AAF disappearance and AAF formation were similar.

Further studies showed that N-OH-GAF inactivation of the cytosolic enzymes was irreversible. After preincubation with N-OH-GAF (20 μM for 60 min), the amount of enzyme activity which remained was appproximately 13% before dialysis and 25% after dialysis (Table 1).

In contrast to the results obtained for cytosolic enzymes, N-OH-AAF and N-OH-GAF were metabolized to a similar extent by microsomal enzymes with no obvious inactivation of the enzyme(s) (Fig. 3). By 120 min, more than 75% of the added substrates was degraded. AAF was not detected as a microsomal metabolite for either substrate. The biotransformation of both N-OH-AAF and

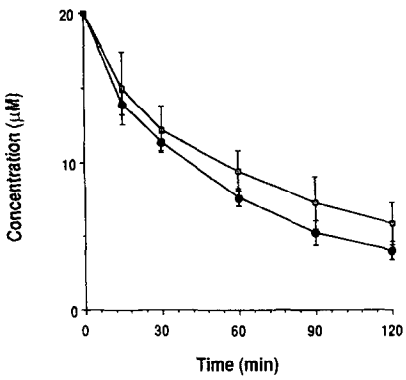

Fig. 3. Time course of N-OH-AAF and N-OH-GAF disappearance in the presence of microsomal enzymes. N-OH-AAF or N-OH-GAF (20 μM) was incubated with 2 mg/ml of microsomal proteins. Aliquots of 0.5 ml were removed at 0–120 min to determine substrate disappearance (□, N-OH-AAF; ●, N-OH-GAF) by HPLC methods. Values plotted are mean ± S.D. from 2 experiments.

N-OH-GAF by both cytosol and microsomal fractions was enzyme mediated, since heat denaturation resulted in a significant decrease of activity (data not shown).

DISCUSSION

Our results showed that the relative rates of biotransformation of the aminofluorene hydroxamic acids, N-OH-GAF and N-OH-AAF, by rat liver cytosolic enzymes were quite different from those by the microsomal enzymes (compare Figs. 1 and 3). While both substrates were rapidly metabolized by microsomal enzymes, there was a significant difference in the rates of cytosolic metabolism of N-OH-AAF and N-OH-GAF. In a 2-h period, N-OH-AAF was also completely metabolized; whereas only 25% of N-OH-GAF was metabolized. The absence of any significant biotransformation of N-OH-GAF after 1 h, even though 75% of the added substrate still remained, indicated that N-OH-GAF might be a suicide substrate. Further studies showed that N-OH-GAF not only inactivated the enzyme(s) involved in its own metabolism, but also those responsible for N-OH-AAF metabolism. N-OH-GAF was found to be a more potent inhibitor of N-OH-AAF metabolism than was N-OH-AAF. The inhibition of N-OH-AAF metabolism by N-OH-GAF was irreversible, as well as time and concentration dependent. These observations, along with the considerations presented by Banks and Hanna [2], suggest that N-OH-GAF is a suicide inhibitor of one or more cytosolic enzymes that are involved in the metabolism of N-OH-AAF.

Based on the direct correlation between the inhibition by N-OH-GAF of N-OH-AAF disappearance and AAF formation, our results suggest that at least one of the target enzyme(s) is a reductase or an enzyme(s) that contributes to the reduction of hydroxamic acids. The presence of a cytosolic hydroxamic acid

reductase was first reported by Gutmann and Erickson [7] and confirmed by Kitamura and Tatsumi [10]. This reductase may be an *N,O*-acyltransferase, since comparison of the purification techniques, elution charactertistics, and molecular weights of the reductase isolated by Kitamura and Tatsumi [10] with the *N,O*-acyltransferase characterized by Allaben and King [1] showed considerable similarity between the two enzymes. The production of AAF might occur indirectly through the action of *N,O*-acyltransferase. The action of this enzyme on N-OH-AAF produces the putative intermediate, *N*-acetoxy-2-aminofluorene, which is thought to be easily reduced by chemical reductants to give 2-aminofluorene (AF) [14]. In turn, AF can serve as an acceptor substrate for the acetyl group from N-OH-AAF through the action of *N,O*-acyltransferase to give AAF. On the other hand, the reactive *O*-sulfate ester produced by the action of sulfotransferase on N-OH-AAF can be directly reduced to the amide, AAF, via a chemical reaction [9].

For comparison, we also studied the glycolyl- and acetyl-derivatives of 4-hydroxylaminobiphenyl, and found that the glycolyl derivative also caused inactivation of cytosolic enzymes (data not shown). The extent of inhibition by *N*-hydroxy-4-glycolylaminobiphenyl was similar to that observed for N-OH-GAF in the present study.

The potent inhibition of N-OH-GAF on N-OH-AAF metabolism may be of toxicological significance since glycolylhydroxamic acids might be formed under physiological conditions [4,5]. Glycolylhydroxamic acids which act as suicide substrates for *N,O*-acyltransferase and/or sulfotransferase might play an important role as inhibitors of the final bioactivation reaction(s) of acetylhydroxamic acids. On the other hand, the overall inhibition of hydroxamic acid reduction to the amide would probably be an undesirable reaction from a toxicological viewpoint. Research is in progress to identify those enzymes involved in hydroxamic acid metabolism which are inhibited by N-OH-GAF.

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