The role of acetylation in the mutagenicity of the antitumor agent, batracylin

Gregory J. Stevens, Edmond J. LaVoie and Charlene A. McQueen

Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721 and Department of Pharmaceutical Chemistry, Rutgers, The State University of New Jersey, Piscataway, NJ 08855, USA

To whom correspondence should be addressed

The role of acetylation in the genotoxicity of the heterocyclic amine, batracylin, was evaluated in Salmonella typhimurium strains expressing various levels of N- and O-acetyltransferase activity. A significant correlation was observed between batracylin-induced mutagenicity and bacterial N-acetyltransferase activity. Strains with the greatest capacity for N-acetylating batracylin (YG 1012 and YG 1024) were the most sensitive to the mutagenic effects of the drug. The number of revertants/nmol batracylin and the formation of acetylbatracylin were ~50-fold greater in YG 1024 compared to TA 98 which expresses endogenous levels of N-acetyltransferase. A similar response was observed with strains YG 1012 and TA 1538. Strains (TA 98/1,8-DNP or TA 1538/1,8-DNP) which lack the ability to N-acetyl batracylin were the least sensitive to the mutagenic effects of the drug. At 1 μg/plate of batracylin, the number of revertants in TA 1538 and TA 98 was 4-fold higher than that observed in TA 1538/1,8-DNP and TA 98/1,8-DNP. To determine if batracylin was a substrate for human N-acetyltransferase, assays were performed in bacteria expressing NAT1 or NAT2. Both strains were capable of N-acetyllating batracylin. The strain expressing NAT2 (DJ 460) formed a significantly greater amount of acetylbatracylin, as well as batracylin-induced revertants, compared to the strain expressing NAT1 (DJ 400). These results demonstrate that the mutagenicity of batracylin is directly related to N-acetyltransferase activity. Data obtained in bacteria expressing either human NAT1 and NAT2 show that batracylin is capable of being bioactivated by both human enzymes. In addition, the higher enzyme activity and mutagenicity in bacteria expressing NAT2 suggests that batracylin is a substrate of this enzyme in humans.

Introduction

Batracylin (BAT*), an experimental antitumor agent, is a potent genotoxicant in bacteria and mammalian cells (1). This compound is a heterocyclic arylamine, structurally related to many of the food mutagens including 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx). Pyrolysate products, such as MeIQx, induce tumors in rodents and form DNA reactive products via the free amine (2,3). Although the carcinogenicity of BAT has not been evaluated, the genotoxic effects of BAT indicate interactions with DNA.

Recently, the chemotherapeutic mechanism of BAT was shown to differ from that leading to genotoxicity (4). Batracylin and several structural analogues were compared for their ability to inhibit DNA topoisomerase II and to elicit unscheduled DNA synthesis in rat hepatocytes (4). Isoindolo[2,1-b]quinazolin-12(10H)-one (8-desaminoBAT), a structural analogue of BAT which lacks the 8-amino group, was not genotoxic in rat hepatocytes. The topoisomerase inhibition and cytotoxicity of 8-desamino BAT were similar to BAT but required higher concentrations. These data suggest that the genotoxic effects of BAT require the free amine while the antimutagen effects are independent of the amino group.

Batracylin is effective in treating early and advanced stage adenocarcinoma 38 in mice (5), as well as murine leukemia P-388 cell lines resistant to cisplatin, adriamycin and methotrexate (5,6). Susceptibility to BAT toxicity was species dependent (7). Rats were extremely sensitive to the drug, compared to mice, while dogs were relatively insensitive. The greater sensitivity of the rat has been associated with a high plasma concentration of the N-acetyl metabolite of BAT. Following administration of equivalent doses of BAT to mice and rats, the plasma concentration of N-acetylbatracylin (ABAT) in rats was 9-fold greater than that in mice (8). It has been suggested that this was due to a difference in the rate of N-acetylation (8). This is consistent with the observation that dogs, a species known to exhibit low N-acetyltransferase (NAT) activity (9), could tolerate a high dose, twice the LD50 of mice (7).

Species differences in the bioactivation and detoxification of many arylamine drugs and carcinogens have been observed (10). The proposed mechanism of bioactivation of heterocyclic amines occurs through initial N-hydroxylation via cytochrome P450 followed by esterification catalyzed by the phase II enzymes, such as UDP-glucuronosyltransferase, sulfotransferase and/or acetyltransferase (NAT/O-acetyltransferase; OAT). Variations in the capacity to acetylate aromatic amine drugs and carcinogens are well-known. A polymorphism in the activity of NAT/OAT has been extensively studied in a number of species including humans (11,12). The enzyme responsible for N-acetylation is the same enzyme as OAT (12). Two isoforms which have NAT and OAT activity have been identified. These enzymes are designated NAT1 and NAT2. The NAT2 enzyme is responsible for the polymorphism identified with substrates such as sulfamethazine. Individuals are classified as rapid and slow acetylators and susceptibility to aromatic amine toxicity has been associated with acetylator phenotype (11).

The involvement of NAT/OAT in the genotoxicity of BAT was evaluated in S.typhimurium strains expressing various levels of the bacterial enzyme responsible for NAT/OAT activity (13-15). Elevated levels of NAT/OAT activity are associated with increased sensitivity to the mutagenic effects.
of many arylamines (16). To determine if human NAT/OATs enhance the mutagenicity of BAT, assays were also performed in bacteria expressing either the human NAT1 or NAT2 enzyme.

Materials and methods

Chemicals

Batracylin (NSC-320846) was obtained from the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) or synthesized (4). Isodiol(1,2,6)-quinazolin-12(10H)-one was prepared as previously described (4). N-acetylbatracylin was made by mixing BAT in pyridine (Fisher Scientific, Fairlawn, NJ) with acetic anhydride (Aldrich, Milwaukee, WI). Aroclor 1254 was obtained from Chem Service (West Chester, PA). HPLC grade solvents were purchased from Fisher Scientific (Springfield, NJ). All other chemicals used were of reagent grade.

Bacterial N-acetyltransferase activity

The ability of each strain to N-acetylate BAT was assessed under conditions that were identical to those used in the mutagenicity assays. Bacteria were grown overnight in nutrient broth until the optical density (OD) reached 1.0 at 650 nm. A 100 μl aliquot of the bacterial suspension was diluted with 500 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 60 mM potassium chloride. Culture tubes were kept on ice until reactions were started with the addition of 50 μl of 10-3 M BAT. Tubes were placed in an orbital shaker (225 r.p.m.) at 37°C for varying times and reactions were terminated by the addition of 600 μl cold methanol. Bacteria were sonicated and centrifuged for 5 min at 5000 g. A 50 μl aliquot of the supernatant was analysed by reverse-phase HPLC using a Spectra-Physics P200. The HPLC conditions used were modified from the method described by Ames et al. (8). Briefly, BAT and ABAT were separated using a C18 (Microsorb-MV 5.6×250 mm, 5 μm) column (Rainin, Woburn, MA) under isocratic conditions. The mobile phase consisted of 75-40 mM ammonium acetate and 25% acetonitrile at 1 ml/min. The absorbance for BAT and ABAT was followed at 413 nm. Under these conditions, retention times were 9.5 and 12.3 min for BAT and ABAT respectively. The amount of ABAT produced at various time points was calculated from a standard curve generated using authentic ABAT and the data were expressed as nmol ABAT/min. For purposes of comparing BAT acetylation to mutagenicity, the rate of BAT acetylation (nmol ABAT/min) was calculated using the linear portion of the time curves.

Mutagenicity assays

Mutagenicity in S. typhimurium was evaluated using the procedures of Maron and Ames (17) as modified for preincubation (18). All strains tested contained the hisD3052 allele and differed only in the presence of the pKM101 plasmid or in NAT/OAT activity. Strains used, their sources and acetylation phenotypes are presented in Table I. The bacterial cultures were grown in nutrient broth until the OD reached 1.0. Ampicillin (25 μg/ml) was included when required. Hepatic 9000 g supernatants were prepared from male Sprague-Dawley rats (Charles River, Kingston, NY) exposed to a single dose of Aroclor-1254 (500 mg/kg i.p.) 5 days before killing. Following a 72 h incubation at 37°C, revertant colonies were counted using an Artek 880 (Dynatech Laboratories Inc., Chantilly, VA). A statistically significant difference in the number of revertants compared to the vehicle control (0.1 ml/plate dimethyl sulfoxide; DMSO) or evidence of a dose-response relationship was necessary for a positive response. Each concentration tested was assayed in triplicate.

Statistics

All comparisons were made using analysis of variance. When appropriate, significance was determined using a Student-Newman-Keuls analysis.

Results

N-acetylation of BAT was assayed to determine the extent of ABAT formation by bacteria expressing either bacterial or human NAT/OAT enzymes. These strains had been previously characterized for N- and/or O-acetylation of aromatic amine carcinogens such as MeIQx, 2-aminofluorene and benzidine (15,19,20). Strains expressing high NAT/OAT activity for these model aromatic amines also had the greatest capacity to N-acetylate BAT. The formation of ABAT in YG 1012 and YG 1024 was linear for 60 min with a rate of 0.51 ± 0.08 and 0.55 ± 0.06 nmol ABAT/min respectively (Table II). In contrast, ~80-fold less of the acetylated metabolite was formed by strains expressing endogenous levels of NAT/OAT (TA 98 and TA 1538). Production of ABAT was linear for 4 h. Activity in TA 98 was 0.009 ± 0.002 nmol ABAT/min and TA 1538 was 0.013 ± 0.002 nmol ABAT/min. No detectable levels of ABAT were observed even after 4 h of incubation with strains lacking NAT/OAT. The formation of ABAT by strains encoding human NAT1 and NAT2 was also evaluated (Table II). In both NAT expressing strains, production of ABAT was linear for 4 h. In the NAT2 expressing strain (DJ 460), there was 0.87 ± 0.003 nmol ABAT/min, compared to 0.030 ± 0.002 in the NAT1 expressing strain (DJ 400). Since these strains lack bacterial NAT/OAT (21), the formation of ABAT was catalyzed solely by the human enzymes.

BAT, in the presence of S9, was mutagenic in several S. typhimurium strains and the number of revertants increased with increasing levels of bacterial NAT/OAT expression. At a concentration of 0.1 μg/plate, BAT induced 1958 ± 329 revertants/plate in YG 1012, a strain overexpressing NAT/OAT, compared to 576 ± 146 revertants/plate in TA 1538 with lower enzyme activity (Figure 2). The strain expressing no NAT activity, TA 1538/1,8-DNP, was the least sensitive, with only 39 ± 5 revertants/nmol at 0.1 μg/plate. Concentrations >0.5 μg/plate of BAT were required to induce a significant increase in the number of revertants in TA 1538/1,8-DNP. A similar response was observed in strains TA 98, TA 98/1,8-DNP, and YG 1024 (Figure 3). The strain having the highest level of NAT/OAT (YG 1024) was the most sensitive to the mutagenicity of BAT. At 0.1 μg/plate, YG 1024 formed 2062 ± 344 revertants/plate, while TA 98 and TA 98/1,8-DNP produced 288 ± 83 revertants/plate and 56 ± 16 revertants/plate respectively. The mutagenicity of BAT was also evident in strains having human NAT1 (DJ 400) and NAT2 (DJ 460).

Table I. Source and characteristics of the S. typhimurium strains tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 1538</td>
<td>hisD3052</td>
<td>B.N.Ames</td>
</tr>
<tr>
<td>TA 1538/1,8-DNP</td>
<td>As TA 1538 but deficient in NAT/OAT</td>
<td>M.Watanabe</td>
</tr>
<tr>
<td>YG 1012</td>
<td>TA 1538/1,8-DNP (pYG213): NAT/OAT overproducing strain</td>
<td>M.Watanabe</td>
</tr>
<tr>
<td>DJ 400</td>
<td>TA 1538/1,8-DNP (pNAT1): human NAT1 expressing strain</td>
<td>P.D.Joseph</td>
</tr>
<tr>
<td>DJ 460</td>
<td>TA 1538/1,8-DNP (pNAT2): human NAT2 expressing strain</td>
<td>P.D.Joseph</td>
</tr>
<tr>
<td>TA 98</td>
<td>hisD3052 (pKM101)</td>
<td>B.N.Ames</td>
</tr>
<tr>
<td>TA 98/1,8-DNP</td>
<td>As TA 98 but deficient in NAT/OAT</td>
<td>E.McCoy</td>
</tr>
<tr>
<td>YG 1024</td>
<td>TA 98 (pYG219): NAT/OAT overproducing strain</td>
<td>M.Watanabe</td>
</tr>
</tbody>
</table>
Table II. Comparison between BAT mutagenicity and the rate of BAT acetylation in *S. typhimurium*

<table>
<thead>
<tr>
<th>Strain</th>
<th>BAT conc. (µg/plate)</th>
<th>Revertants/nmol</th>
<th>nmol ABAT/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 98</td>
<td>0.05</td>
<td>1016 ± 136</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>TA 1538</td>
<td>0.05</td>
<td>1594 ± 398</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>YG 1012</td>
<td>0.001</td>
<td>37 351 ± 7437</td>
<td>0.511 ± 0.08</td>
</tr>
<tr>
<td>YG 1024</td>
<td>0.001</td>
<td>49 863 ± 14 243</td>
<td>0.547 ± 0.06</td>
</tr>
<tr>
<td>DJ 400</td>
<td>0.05</td>
<td>413 ± 165</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>DJ 460</td>
<td>0.05</td>
<td>613 ± 196</td>
<td>0.087 ± 0.003</td>
</tr>
</tbody>
</table>

A significant correlation (*P* < 0.001, *r*² = 0.97) was observed between the number of revertants/nmol versus nmol ABAT/min.

*Concentration used to calculate number of revertants/nmol.

*Data represent the mean ± SD (n = 3 or 4).

The presence of either human NAT resulted in a greater number of mutants at both 0.5 and 1.0 µg/plate compared to the parental strain, TA 1538/1,8-DNP₆ (●) and YG 1012 (■). All incubations were performed in the presence of Aroclor-induced rat liver S9. Each concentration was performed in triplicate and expressed as the mean ± SD of three independent experiments. Revertants/plate in the presence of DMSO were 35 ± 9, 40 ± 7 and 130 ± 16 for TA 98, TA 98/1,8-DNP₆ and YG 1024 respectively.

The mutagenic potential of ABAT was evaluated in TA 98. In the absence of S9, a concentration dependent increase in the number of revertants occurred above 1.0 µg/plate (Figure 5). At 250 µg/plate, the highest concentration tested, ABAT induced 1764 ± 327 revertants/plate. Although revertants were observed without S9, the mutagenicity of ABAT was dramatically increased by the addition of mammalian biotransformation enzymes. At a concentration of 1 µg/plate, 2004 ± 198 revertants/plate were observed with S9 compared to 76 ± 3 revertants/plate without.

For each bacterial strain, the highest number of revertants/nmol was calculated and compared to BAT NAT activity (Table II). Strains YG 1024 and YG 1012, which expressed the highest rate of BAT acetylation were also the most sensitive to the mutagenic effects of BAT.

**Discussion**

Batracyclin is structurally similar to carcinogenic aromatic and heterocyclic amines. Metabolism of this class of compounds is complex and involves both phase I and phase II enzymes. A major route of bioactivation of aromatic amine carcinogens is believed to occur through *N*- and/or *O*-acetylation of the
amino resulting in the formation of arylnitrenium ions (22–24). The highly reactive nitrenium ions interact with cellular nucleophiles such as DNA and protein. Although BAT was genotoxic and acetylation has been implicated in structure-activity studies (4), the role of NAT/OAT in the mutagenicity of BAT has not been described.

A previous study of the mutagenicity of BAT in *S. typhimurium* was extended to strains with varying capacities for acetylation. Those strains producing the most ABAT were the most sensitive to BAT mutagenicity (Table II). YG 1024 was 50-fold more sensitive to the mutagenic effects of BAT than TA 98. There were 1016 revertants/µmol compared to 49 863 revertants/µmol produced in the YT 1024 strain, while the strains lacking NAT/OAT (TA 98/1,8-DNP) had <200 revertants/µmol. Similarly, the amount of ABAT produced by YG 1024 was 60-fold higher than TA 98 and no ABAT was detected in those incubations with strains lacking NAT/OAT activity. These data support the hypothesis that NAT/OAT is involved in the mutagenicity of BAT. The relative contributions of N- or O-acetylation to the mutagenicity of BAT are still to be determined. It is known that the same protein in mammals catalyzes three acetylation reactions: N-acetylation of arylamines, O-acetylation of N-hydroxyarylamines and transfer of the acetyl group from N to O of arylhydroxamic acids (11). Studies with other heterocyclic amines implicate O-acetylation as a critical step in the mutagenicity in *S. typhimurium* (24).

An S9 activating system was generally required for the mutagenicity of BAT; although, two exceptions were noted. First, BAT induced revertants in YG 1012 and YG 1024, the NAT/OAT overexpressing strains, without S9; however, the number of revertants was 10-fold less than with S9 (data not shown). A similar response was observed in YG 1012 with benzidine (19). Secondly, ABAT was a direct acting mutagen capable of inducing revertants in TA 98 without the addition of S9. Again, a greater number of revertants was induced when S9 was present. This suggests that other bioactivation steps contribute to the genotoxicity of BAT. While NAT/OAT plays a major role in the mutagenicity of BAT, revertants were observed in TA 98/1,8-DNP6 and TA 1538/1,8-DNP6 despite the lack of detectable levels of ABAT. This is consistent with a bioactivation pathway independent of NAT/OAT. One possibility is N-hydroxylation of BAT, since other N-hydroxy heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) are direct acting mutagens (25,26). The extent of N-hydroxyBAT formation is currently under investigation.

Several lines of evidence have led to the conclusion that acetylation is a critical step in the bioactivation of BAT. Species differences observed in the adverse effects of BAT appear to be associated with differences in NAT/OAT activity. *In vivo*, rats were more sensitive to BAT toxicity and had higher plasma levels of ABAT than mice, the less sensitive species (8). *In vitro*, rat (F-344) hepatocytes were found to be more susceptible to the genotoxic effects of BAT compared to mouse (C-57Bl/6J) cells (1). In addition, the rate of BAT acetylation in rat hepatic tissue was 6-fold higher than that of mouse liver (27). This suggests that either N- and/or O-acetylation of BAT differs from that of other aromatic and heterocyclic amines, which are generally poor substrates of rodent acetyltransferases (28). Finally, the present study reports a strong correlation between BAT acetylation and bacterial mutagenicity.

These results from animal and *in vitro* studies implicate acetylation in the human toxicity of BAT, a potential cancer chemotherapeutic agent. In humans, acetylation of aromatic amines is catalyzed by two enzymes, NAT1 and NAT2. The latter enzyme is responsible for the human polymorphism which has been associated with susceptibility to drug toxicity (11,29). One example of enhanced toxicity due to activation via N-acetylation was observed with the chemotherapeutic agent amonafide (30,31). Patients classified as rapid acetylators were found to have a significantly greater myelosuppression following amonafide treatment than did slow acetylators (30).

The ability of human NAT1 and/or NAT2 to acetylate BAT was investigated in *Salmonella* strains that lack bacterial NAT but express either human NAT1 or NAT2 (24). The number of revertants seen when acetylation was catalyzed by the strains expressing either of the human enzymes (DJ 400 and DJ 460) was lower than with TA 1538, expressing the bacterial enzyme (Figure 4). This may result from kinetic differences between human and bacterial enzymes. Another possibility is that the expression of the NAT/OAT gene varies among these three strains. Previous work has suggested that the bacterial NAT/OAT enzyme may have a greater ability to O-acetylate N-hydroxyarylamines than the human enzymes (21). Comparison of the two strains with the human enzymes reveals that DJ 460 (NAT2) had a significantly higher rate of BAT acetylation and more revertants than DJ 400 (NAT1) (Figure 4 and Table II). Differences in revertant frequencies between DJ 400 and DJ 460 have been observed with several other aromatic and heterocyclic amines (20,21). A recent report suggests NAT1 lacks the ability to O-acetylate heterocyclic hydroxylamines (22), which may account for the higher mutagenic response of BAT in DJ 460.

The positive responses induced in the DJ strains demonstrate that the human enzymes are capable of activating BAT and suggest BAT is a substrate of the polymorphic NAT2 in humans. Consequently, rapid acetylators may be at greater risk of toxicity if exposed to this drug than slow acetylators. The precise kinetics of BAT acetylation in human tissue is currently under investigation.

**Fig. 5.** Mutagenic activity of ABAT in strain TA 98 in the presence (■) and absence (□) of Aroclor-induced rat liver S9. Each concentration was performed in triplicate and data expressed as the mean ± SD of three independent experiments. Revertants/plate in the presence of DMSO were 34 ± 2 in the absence of S9 and 67 ± 19 in the presence of S9.
Acknowledgements

GJS was supported by NIH training grant ES07091 and a Procter and Gamble pre-doctoral fellowship. Studies were supported by grants ES-05174 and Center Grant IP30 ES06694 from the National Institute of Environmental Health Sciences.

References


Mutagenicity of batracylin