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N-Alkylaminobenzotriazoles as Isozyme-Selective Suicide Inhibitors of Rabbit Pulmonary Microsomal Cytochrome P-450

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SUMMARY

To produce potent, isozyme-selective suicide inhibitors of cytochrome P-450 (P-450), a series of *N*-alkylated 1-aminobenzotriazole (ABT) derivatives was synthesized; these included the *N*-methyl, *N*-butyl (BuBT), *N*-benzyl (BBT), and *N*-α-methylbenzyl (αMB) analogues of ABT. The suicide inhibitors showing the greatest potency and isozyme selectivity were BBT and αMB, compounds which included molecular features for P-450 inactivation (the ABT moiety) and similarity to benzphetamine. ABT and its *N*-alkylated derivatives were tested as suicide inhibitors in rabbit lung microsomes, whose P-450 monooxygenase system has been well characterized in both untreated and β-naphthoflavone- or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated animals. ABT (10 mM) destroyed up to 99% of the total P-450 content of lung microsomes of untreated rabbits. At equimolar concentrations (10 μM), ABT was less effective than the *N*-alkylated compounds for the inhibition of P-450 isozyme 2-catalyzed benzphetamine *N*-demethylation (BND); in fact, BuBT, BBT, and αMB completely inhibited BND activity at this concentration and destroyed less than 40% of total pulmonary P-450. However, these compounds also inactivated 69–85% of isozyme 6-catalyzed 7-ethoxyresorufin *O*-deethylation. The most potent and isozyme-selective suicide inhibitor prepared was αMB: at 1 μM this compound inhibited approximately 80% of isozyme 2-catalyzed and 20% of isozyme 6-catalyzed monooxygenase activity but spared P-450 isozyme 5; at 2.5 μM it caused a near-complete loss (96 ± 2%) of BND activity. The partition ratio of αMB, i.e., the molar ratio of inhibitor present to that of the P-450 destroyed, was 11 ± 2, further demonstrating the potency of this compound. Experiments with BBT- and sodium phenobarbital-treated rats showed that the mechanism for suicidal inactivation of P-450 by this *N*-alkylated compound was by benzyne release, the same mechanism demonstrated earlier for the parent compound ABT.

The P-450-dependent monooxygenase system plays a central role in the metabolism of xenobiotics and also participates in the biosynthesis and catabolism of several essential endogenous compounds. The products of these transformations can be of equal, greater, or lesser biological activity or toxicity than the parent compound. Although the highest microsomal monooxygenase activities are normally found in the liver, the lung also contains an active P-450 system. The rabbit lung contains three P-450 isozymes, forms 2, 5, and 6. Forms 2 and 5, which each constitute 30–40% of total lung P-450, are also found in liver and are indistinguishable from those isozymes induced in liver after treatment with PB (1–3). However, pulmonary forms 2 and 5 are not induced by PB, although the overall ratios of P-450 isozymes change slightly in response to PB due to selective repression of isozyme 6 (3). Form 6 is induced by polycyclic aromatic hydrocarbon-type compounds, including 3-methylcholanthrene, β-NF, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, in both rabbit lung and liver, whereas form 4 is induced only in rabbit liver (3–5). Maximal induction by β-NF treatment results in an increase in the specific content of form 6 in untreated pulmonary microsomes from 0.5–5.0% of total P-450 to as much as 20% of total lung P-450 (5). Specific monooxygenase assays are known for two of these isozymes in rabbit lung; the *N*-demethylation of benzphetamine (BND) is catalyzed only by form 2 and the *O*-deethylation of 7-ethoxyresorufin (7-ERF) is catalyzed only by form 6 (3). However, both forms 4 and 6 catalyze the *O*-deethylation of 7-ERF in liver.

ABT is a suicide substrate for a wide variety of P-450 isozymes derived from lung (6), liver (7, 8) and plant (9) sources. The enzymatic oxidation and the chemical oxidation (10) of ABT yield the reactive benzyne species. The intermediacy of benzyne in the inactivation of P-450 was demonstrated by the isolation of an *N,N'*-bridged porphyrin adduct (7, 11). Subse-

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ABBREVIATIONS: P-450, cytochrome P-450; ABT, 1-aminobenzotriazole; αMB, *N*-α-methylbenzylaminobenzotriazole; BBT, *N*-benzylaminobenzotriazole; BND, benzphetamine *N*-demethylase; β-NF, β-naphthoflavone; BuBT, *N*-butylaminobenzotriazole; DETAPAC, diethylenetriaminepentaacetic acid; DMA, *N,N*-dimethylaniline; 7-ERF, 7-ethoxyresorufin *O*-deethylase; FCM, flavin-containing monooxygenase; GSH, glutathione; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LPC, low pressure chromatography; MBT, *N*-methylaminobenzotriazole; PB, sodium phenobarbital; SO, styrene 7,8-oxide.

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quently, it was found that *N*-alkyl derivatives of ABT are also suicide substrates of P-450 (11).

The primary objective of this study was to produce isozyme-selective inhibitors of rabbit P-450 by designing compounds that include molecular features for enzyme destruction and mimicry of known isozyme-selective substrates. Such compounds have potential for use as specific inhibitors of different P-450 isozymes in intact cells and *in vivo* (12–14). Three of the compounds synthesized, BuBT, BBT, and αMB (Fig. 1), were shown to be potent suicide inhibitors of pulmonary P-450 form 2 with considerable selectivity for this isozyme.

Materials and Methods

Synthesis of ABT and its *N*-alkylated derivatives. ABT, *N*-acetyl-ABT, and MBT were synthesized as previously described (11). *N*-Alkyl derivatives were prepared by formation and reduction of Schiff's bases (method A) by an adaptation of the procedure of Somei *et al.* (15) or by alkylation of *N*-acetyl-ABT with the appropriate alkyl halide followed by amide hydrolysis (method B) (11). All reactions were performed under a nitrogen atmosphere. Reagents used for synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI).

1-Benzylideneaminobenzotriazole and BBT (method A). To a solution of ABT (840 mg, 6.27 mmol) in glacial acetic acid (100 ml), benzaldehyde (3.8 g, 35.8 mmol) was added. After stirring overnight at room temperature, the mixture was evaporated *in vacuo* to leave an oil. This residue was dissolved in 70 ml of CH_2Cl_2/MeOH (95:5) and washed three times with 50 ml of 5% sodium bicarbonate and then with three 40-ml portions of water. The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo* to yield 3.01 g of crude product. To 2.62 g of this product in 300 ml of MeOH/CH_2Cl_2 (2:1) was added $NaBH_4$ (19.61 g, 518 mmol). After stirring for 1 hr at room temperature, the solvent was evaporated to leave a white solid which was dissolved in 300 ml of CH_2Cl_2/MeOH (19:1). The organic phase was washed with three 150-ml portions of water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product, purified by LPC (hexane/ethyl acetate; 1:1, v/v), gave 1.09 g (78%) of BBT as white crystals, m.p. 93–94.5° (lit. 89–90°, Ref. 16). NMR ($CDCl_3$) 1.5–1.8 (1H, broad s, amine, exchangeable with CD_3OD), 4.57 (2H, s, benzyl), 7.26–7.41 (8H, m, phenyl) and 7.96–7.98 ppm (1H, d, phenyl proton at C-7 of benzotriazole). Exact mass (*m/z*): 224.1062 (M^+) expected for $C_{13}H_{12}N_4$, 224.1055 found (2.9 ppm deviation).

In the same fashion BuBT was synthesized in 64% yield after reduction of the Schiff's base formed from the condensation of ABT with butyraldehyde, and subsequent purification by LPC (chloroform/ethyl acetate; 1:3, v/v) as a colorless liquid. NMR ($CDCl_3$) 0.87–0.95 (3H, t, methyl), 1.39–1.65 (4H, m, methylene), 3.39–3.44 (2H, m, methylene α to amino), 4.11–4.15 (1H, br m, amino), 7.34–7.63 (3H, m, phenyl), and 8.00–8.03 ppm (1H, d, phenyl proton at benzotriazole C-7). Exact mass (*m/z*): 191.1295 (M + H) expected for $C_{10}H_{10}N_4$, 191.1300 found (2.6 ppm deviation).

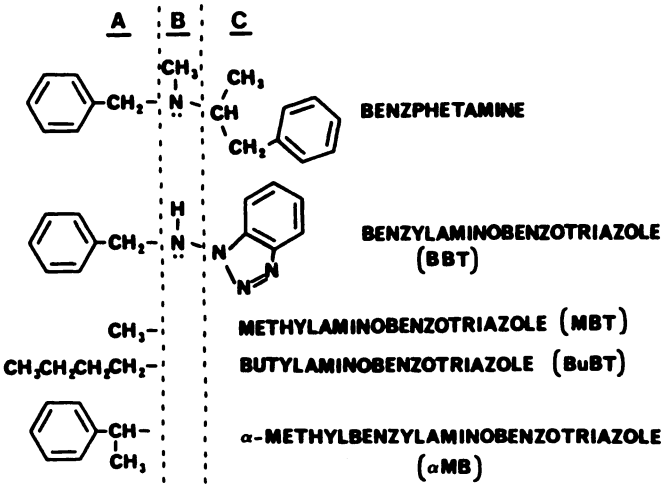

Fig. 1. Design of the suicide substrates BBT and αMB to mimic benzphetamine in containing (A) a benzyl group, (B) an amine group in the desired region of oxidation, and (C) an aromatic region of similar dimension.

αMB. *N*-acetyl-ABT (1.31 g, 7.46 mmol) was added to a suspension of 3.0 g of potassium carbonate in 50 ml of 2-pentanone. The solution was heated to reflux and (1-bromoethyl)benzene (2.03 g, 11.0 mmol) was added. After 24 hr the solution was cooled, filtered, and concentrated. The oily residue, purified by LPC (hexane/ethyl acetate, 1:1, v/v), yielded 1.69 g (81%) of *N*-acetyl-αMB. Methanolic potassium hydroxide solution (5 M) was added to this amide and the solution was heated to reflux for 2 hr, cooled, and concentrated. The residue was extracted into 50 ml of CH_2Cl_2, and the organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated. The oily residue, purified by LPC (hexane/ethyl acetate, 1:1) gave αMB (1.31 g, 91%) as white crystals (HCl salt, m.p. 96–98.5°). NMR ($CDCl_3$) 1.43–1.45 (3H, d, methyl), 4.88–4.95 (1H, q, benzyl), 5.0–5.5 (1H, broad s, amine), 7.23–7.50 (8H, m, phenyl), and 7.94–7.97 ppm (1H, d, phenyl proton at C-7 of benzotriazole). Exact mass (*m/z*): 239.1297 (M + H^+) expected for $C_{14}H_{15}N_4$ 239.1253 found (18 ppm deviation).

Animals and treatment. Male New Zealand White rabbits (2–3 kg) were used. Some rabbits were treated intraperitoneally with β-NF (80 mg/kg) in corn oil on days 1 and 2. These animals were sacrificed on day 5 by asphyxiation with CO_2 just prior to surgery. Other rabbits were treated intraperitoneally with PB in saline, 50 mg/kg, once daily for 3 days, with the last dose being injected 24 hr before death. The male Sprague-Dawley rats used (250–350 g) were injected intraperitoneally once daily for 5 days (50 mg/kg) with an aqueous solution of PB (50 mg/ml) prior to death. All animals were allowed free access to food and water throughout the treatment period.

Reagents. [^{14}C]Styrene (^{14}C-ring, 1.83 Ci/mol, 97% radiochemical purity) was purchased from Amersham (Arlington Heights, IL) and diluted with unlabeled styrene from Aldrich Chemical Co. Aquasol liquid scintillation cocktail was supplied by New England Nuclear (Boston, MA). β-NF, NADPH, GSH, HEPES, and DETAPAC were purchased from Sigma Chemical Co. (St. Louis, MO), 7-ERF was obtained from Pierce Chemical Co. (Rockford, IL), and *d*-benzphetamine hydrochloride was a generous gift from The Upjohn Company (Kalamazoo, MI). All other reagents were of the highest grade commercially available.

Effect of inhibitor concentration on the "destruction" of total P-450, and inhibition of BND and 7-ERF activities in pulmonary microsomes from rabbits treated with β-NF. Lung microsomes were prepared as previously described (6). Incubation mixtures contained pulmonary microsomal protein (15 mg), NADPH (15 μmol), and inhibitor (no inhibitor in controls) in 15 ml of 0.1 M potassium phosphate buffer, pH 7.4. Lipophilic inhibitors (MBT, BuBT, BBT, αMB) were dissolved in methanol and added to the incubation flask. The solvent was totally removed under a gentle stream of N_2 prior to addition of other incubation components. After 45 min at 37°, the incubations were cooled on ice, adjusted to a final volume of 28 ml with phosphate buffer, and centrifuged for 30 min at 100,000 × *g*. The pellets were washed by resuspension and resedimentation to remove inhibitor.

We demonstrated earlier (6) that such a washing step with pulmonary microsomes decreases the ^{14}C-ABT concentration to a maximum value of 0.6 μM in the subsequent BND and 7-ERF assays, and that this level of suicide inhibitor affects neither BND nor 7-ERF activity. Similar experiments with unlabeled BBT (5 μM) showed minor decreases of BND activity (about 10%) and 7-ERF activity (up to 20%) in pulmonary microsomes that were first incubated with BBT in the absence of NADPH versus microsomes incubated only with NADPH prior to measurement of monooxygenase activity. These decreases in monooxygenase activity are due to suicide inactivation by a small

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amount of residual BBT in the washed microsomes and/or competitive inhibition of benzphetamine demethylation by BBT. However, the inhibition by residual inhibitor is minor in comparison to the suicide inactivation due to initial incubation of microsomes with BBT and NADPH (80% loss of BND and 90% loss of 7-ERF activities).

The microsomal pellets were resuspended to a protein concentration of approximately 10 mg/ml, frozen in liquid nitrogen, and stored at −70°. Specific content of P-450 and specific BND and 7-ERF activities were measured subsequently in thawed samples.

Effect of ABT and BBT on the stereoselectivity of the P-450-dependent oxidation of styrene. Incubation mixtures contained pulmonary microsomal protein (10 mg), NADPH (10 μmol), and ABT or BBT (1 μmol) (no ABT or BBT in controls) in 10 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mM $MgCl_2$. After 30 min at 37°, the incubations were cooled in ice, adjusted to a final volume of 28 ml in a centrifuge tube, and centrifuged for 30 min at 100,000 × *g*. Prior to incubation with [^{14}C]styrene, the pellet was washed by resuspension and resedimentation to remove inhibitor.

A standard styrene monooxygenase incubation mixture (1.5 ml) contained the following components in 0.1 M HEPES, pH 7.4: $MgSO_4$ (5 mM), GSH (5 mM), microsomal protein (0.5 mg), NADPH (2 mM), and 20 μg of rat hepatic GSH transferases (Sigma; EC 2.5.2.18, 108 units/mg; 1 unit catalyzes the reaction of 1 μmol of 1-chloro-2,4-dinitrobenzene with GSH per min at 25°, pH 6.5). Boiled microsomes were used as a blank. Reactions were initiated by addition of styrene (2 mM, 1.6 μCi) and terminated after 20 min at 37° by addition of petroleum ether (5 ml) with vortexing. Subsequent to extraction three times with 5 ml of petroleum ether, 1 ml of methanol was added to the aqueous residue in each tube, which was centrifuged to remove protein. The supernatant fractions were analyzed by high pressure liquid chromatography for the two pairs of diastereomeric GSH adducts of *R*- and *S*-SO, which accounted for 95% of the styrene metabolized, as described earlier (17).

***In vitro* destruction of rat hepatic P-450 and heme by BBT and αMB.** Washed hepatic microsomes from PB-treated rats were prepared as previously described (8). The inhibitor analogue (final concentration 0.50 mM) dissolved in methanol was added to an Erlenmeyer flask (25 ml), and the methanol was removed under a stream of nitrogen at room temperature. To the flask were added hepatic microsomal protein (10 mg), NADPH (10 μmol), and DETAPAC (10 μmol) in 10 ml of 0.1 M potassium phosphate buffer, pH 7.4. After incubating for 30 min at 37°, the reaction mixture was cooled in ice. P-450 concentrations were determined as before, using an incubated sample from which NADPH was deleted as control. Heme content was measured by the pyridine hemochromogen method using an extinction coefficient of 34.4 $cm^{-1}mM^{-1}$ for the 557–600-nm absorbance difference (18).

Hepatic pigment isolation and characterization. PB-treated rats were injected intraperitoneally with BBT (400 mg/kg) in dimethyl sulfoxide (200 μl). The rats were decapitated 12 hr later; livers were perfused *in situ* with ice-cold 0.9% NaCl solution and then excised. Pigments were isolated as previously described (11).

Enzyme assays. P-450 concentration was determined from the dithionite difference spectrum of carbon monoxide-saturated microsomes (19) using an Aminco DW-2A spectrophotometer (ε = 100 $mM^{-1}cm^{-1}$). *N*-Demethylation of *d*-benzphetamine (2 mM) was determined by assaying the amount of formaldehyde produced in 10 min at 37° (20). 7-ERF activity was determined by the method of Burke and Mayer (21) as modified by Norman *et al.* (4). FCM activity was measured by oxidation of DMA. Incubation mixtures contained (in a volume of 1.20 ml) 0.1 M Tris-glycine buffer, final pH 8.8, 0.50 mg of microsomal protein, 5 mM glucose 6-phosphate, 1 mM NADP, 1 unit of yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49; 1 unit reduces 1 μmol of NADP/min at 25°), inhibitor, and 2.5 μmol of DMA. The reactions were stopped after 15 min by addition of 0.50 ml of 8.9% $ZnSO_4$, 0.75 ml of saturated $Ba(OH)_2$, and 0.25 ml of saturated $Na_2B_4O_7$ followed by centrifugation. An aliquot of the supernatant was assayed for DMA-*N*-oxide by the method of Ziegler and Pettit (22).

Protein concentrations were determined by the method of Lowry *et al.* (23) using bovine serum albumin as standard.

Results

Effect of ABT concentration on "destruction" of P-450 in pulmonary microsomes from untreated rabbits. As shown in Fig. 2, a linear relationship resulted when the percentage loss of spectrally determined P-450 in pulmonary microsomes was plotted against the log of the ABT concentration. The lowest concentration of ABT at which significant losses of P-450 (28 ± 4%) were measured was 10 μM, and virtually all of the P-450 (99 ± 1%) was destroyed by 10 mM ABT. No losses of P-450 occurred in identical incubation mixtures lacking the monooxygenase cofactor, NADPH.

Comparison of the effects of ABT and its *N*-alkyl analogues on P-450 content and monooxygenase activities of pulmonary microsomes from β-NF-treated rabbits. The potency and selectivity of ABT and its *N*-methyl, *N*-butyl, *N*-benzyl, and *N*-α-methylbenzyl analogues were compared at an inhibitor concentration of 10 μM. ABT proved to be the least effective compound in the series in terms of its ability to inhibit BND activity; BuBT, BBT, and αMB were all more potent in this respect than MBT, which was intermediate in effect (Table 1). Microsomes incubated with BuBT, BBT, or αMB lost all detectable BND activity (catalyzed by P-450 form 2) and most (69–85%) of the form 6-catalyzed 7-ERF, yet they lost less than 40% of the total P-450. Greatly diminished turnover numbers for 7-ERF and BND activities in BuBT- and BBT (but not ABT)-treated microsomes reflect the apparent selectivity and potency of these analogues in inactivation of pulmonary P-450, forms 2 and 6.

Effect of MBT on the P-450 monooxygenase system of pulmonary microsomes from β-NF-treated rabbits. Since 10μM MBT inactivated only about 25% of total P-450 and 70% of BND activity, additional experiments were per-

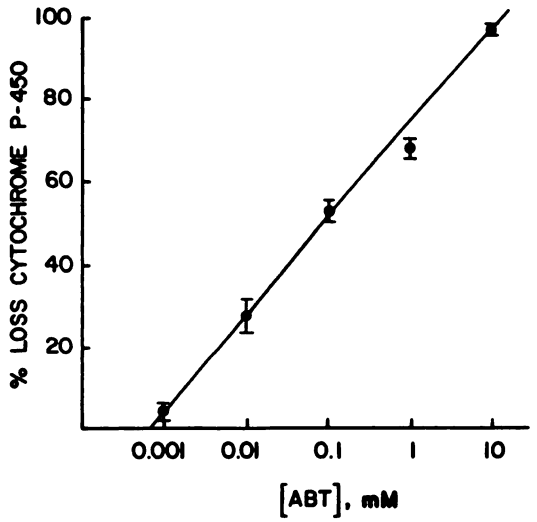

Fig. 2. Percentage loss of P-450 in rabbit pulmonary microsomes (approximately 1 mg of protein/ml) after 30 min incubation with 1 mM NADPH at 37°. One hundred per cent value for P-450 content was 0.31 ± 0.08 nmol of P-450/mg of microsomal protein. Data plotted are means ± standard deviation, *N* = 3 sets of microsomes prepared from the lungs of individual animals.

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TABLE 1

Comparison of the effects of equimolar amounts (10 μM) of ABT and its *N*-alkyl analogues on the P-450 monooxygenase system of pulmonary microsomes from β-NF-treated rabbits[a]

Inhibitor	Percentage loss of		
	P-450 content	BND activity	7-ERF activity
ABT	22 ± 1[b,c]	26 ± 10	18 ± 12
MBT	24 ± 5	69 ± 14	23 ± 10
BuBT	37 ± 8	>99[d]	85 ± 5
BBT	30 ± 2	>99	85 ± 10
αMB	37 ± 2	99 ± 1	69 ± 2

[a] Rabbits were treated intraperitoneally with β-NF, 80 mg/kg, on days 1 and 2 and were killed on day 5.
[b] Values are means ± standard deviation, $N = 3$.
[c] This table contains data that were obtained with several different sets of pulmonary microsomes from individual β-NF-treated rabbits; all compounds could not be tested with any single set of microsomes.
[d] No remaining activity was detected.

TABLE 2

Effect of various concentrations of MBT on the P-450 content and BND and 7-ERF activities of pulmonary microsomes from β-NF-treated rabbits[a]

MBT concentration	Percentage loss of		
	P-450 content	BND activity	7-ERF activity
10 μM	24.2 ± 5.1[b]	69.0 ± 14.1	23 ± 10
1 mM	46.9 ± 8.6	64.2 ± 8.3	69 ± 4
10 mM	54.9 ± 26.0	79.9 ± 14.7	70 ± 10

[a] Rabbits were treated intraperitoneally with β-NF, 80 mg/kg, on days 1 and 2 and were killed on day 5.
[b] Values are means ± standard deviation, $N = 3$. Control (100%) values for P-450 content and for BND and 7-ERF activities were 0.51 ± 0.08 nmol/mg of protein, 10.4 ± 1.6 nmol/min/mg of protein, and 67 ± 26 pmol/min/mg of protein, respectively.

formed with 100-fold and 1000-fold higher inhibitor concentrations (Table 2). At the highest concentration studied (10 mM), inactivation of forms 2 and 6 (80% and 70%, respectively; Table 2) trailed behind that caused by 10 μM BuBT or BBT (100% and 85%, respectively); as the near-complete inactivation of forms 2 and 6 by BuBT and BBT entailed a <40% loss of total P-450, the loss of P-450 caused by MBT at 1 mM and 10 mM (47% and 55%, respectively) suggests that this agent causes some inactivation of form 5 as well, as we previously demonstrated for ABT (6).

Effect of various concentrations of BBT on the P-450 monooxygenase system of pulmonary microsomes from β-NF-treated rabbits. Incubation of microsomes with 1 mM NADPH and varying concentrations of BBT caused parallel losses in activity of forms 2 and 6 throughout the concentration range (100 nM–1 mM) tested (Fig. 3). The percentage loss of P-450 increased linearly with inhibitor concentration up to 1 μM. The partition ratio (molar ratio of inhibitor present to P-450 destroyed) was calculated for this section of the curve to be 14 (in three separate determinations, without variance). At 1 mM, >99% loss of BND and 7-ERF activities occurred, yet loss of total P-450 (41.2 ± 7.4%) was not significantly increased over that found at 10 μM (38.8 ± 6.2%), suggesting an intransigence of form 5 to destruction by BBT.

Effect of ABT and BBT on the stereoselectivity of the P-450-dependent oxidation of styrene by pulmonary microsomes. The stereoselective oxidation of styrene to SO was studied with pulmonary microsomes from PB-treated rabbits where the contribution of form 6 is negligible. The inhibitor concentration chosen (100 μM) produced essentially equal

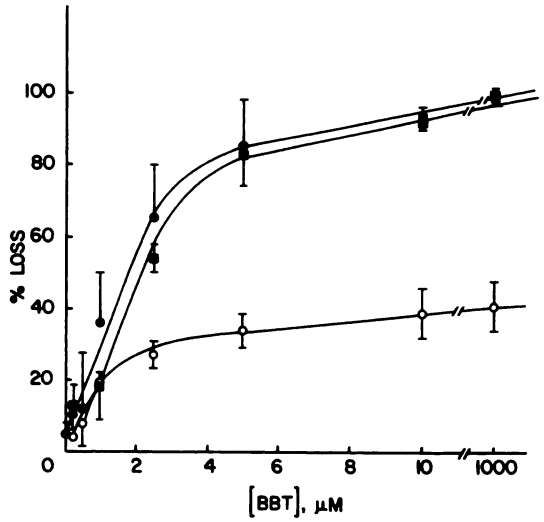

Fig. 3. Percentage loss of P-450 content and BND or 7-ERF activity in pulmonary microsomes (approximately 1 mg of protein/ml) from β-NF-treated rabbits after incubation with BBT and 1 mM NADPH for 45 min at 37°. One hundred per cent values for P-450 content (○), BND (●), and 7-ERF *O*-deethylase (■) activities were 0.46 ± 0.04 nmol/mg of microsomal protein. 8.9 ± 1.3 nmol/mg of microsomal protein/min, and 73 ± 17 pmol/mg of microsomal protein/min, respectively. Data plotted are means ± standard deviation, $N = 3$ where each N represents a pool of lungs from 9–12 rabbits.

losses of P-450 (~40%; Table 3) by ABT and BBT and thus provided an appropriate point at which to further compare the selectivity of isozyme inactivation by these two compounds. We previously demonstrated in reconstituted monooxygenase systems that form 2 selects for the formation of *R*- to *S*-SO by a ratio of 2:1, whereas form 5 shows no selectivity ($R/S = 1.0$) (17). In "control" pulmonary microsomes (i.e., no incubation with ABT or BBT), we found the R/S ratio for the oxidation of styrene to SO to be 1.5. Although both inhibitors caused a 40% decrease in total P-450, ABT caused a shift of the R/S ratio for styrene oxidation to 1.7 (preferential inactivation of form 5) with only a 23% loss of BND activity. The BBT-treated microsomes had an *R*-SO/*S*-SO value of 0.9, and BND activity was decreased by 93%, indicative of near-total inactivation of form 2-mediated activity. However, the catalytic characteristics of form 5 were retained. These data are consistent with our earlier study of ABT-mediated inactivation of P-450 in the perfused rabbit lung (6).

Effect of various concentrations of αMB on the P-450 monooxygenase system of pulmonary microsomes from β-NF-treated rabbits. αMB proved to be the most potent analogue of this synthetic series in the inhibition of BND activity (Fig. 4); a concentration of only 2.5 μM caused near-complete (96 ± 2%) loss of activity. The partition ratio, calculated for the linear section of the curve at and below 1 μM, was 11 ± 2. αMB was also the most selective derivative synthesized. Thus, at 1 μM a preferential inactivation of form 2 (~80%) over form 6 (~20%) was evident. At 1 mM, inactivation of forms 2 and 6 was virtually complete, but the remainder of the P-450, 60% of the total (mostly form 5), resisted inactivation by αMB.

Effect of BBT and αMB on the relative concentrations of P-450 and heme in hepatic microsomes from PB-treated rats. Incubation of rat hepatic microsomes with 0.5 mM BBT or αMB (and NADPH) caused equimolar losses of P-450 and heme (Table 4). The 40–50% destruction of total P-

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TABLE 3

Effect of preincubation with ABT (100 μM) or BBT (100 μM) on the rate and stereoselectivity of the P-450-dependent oxidation of styrene by pulmonary microsomes from PB-treated rabbits[a]

Microsomal source	P-450 content	BND	Stereoselectivity (*R*-SO/*S*-SO)	Specific activity
	nmol/mg protein	*nmol/min/mg protein*		*nmol SO formed/min/nmol P-450*
Control (no inhibitor)	0.45 ± 0.05[b]	8.3 ± 1.3	1.5 ± 0.04	6.1 ± 0.3
ABT	0.26 ± 0.01 (−42.2%)[d]	6.4 ± 1.0 (−22.9%)	1.7 ± 0.1[c] (+13.3%)	6.5 ± 1.2 (+6.6%)
BBT	0.27 ± 0.03 (−40.0%)	0.6 ± 0.6 (−92.8%)	0.9 ± 0.1 (−40.0%)	2.6 ± 0.2 (−57.4%)

[a] Rabbits were treated intraperitoneally with PB, 50 mg/kg, once daily for 3 days; the animals were killed 24 hr after the last dose.
[b] Values are means ± standard deviation, $N = 3$.
[c] We previously reported this value (6).
[d] Values in parentheses, per cent change (of mean) from control value.

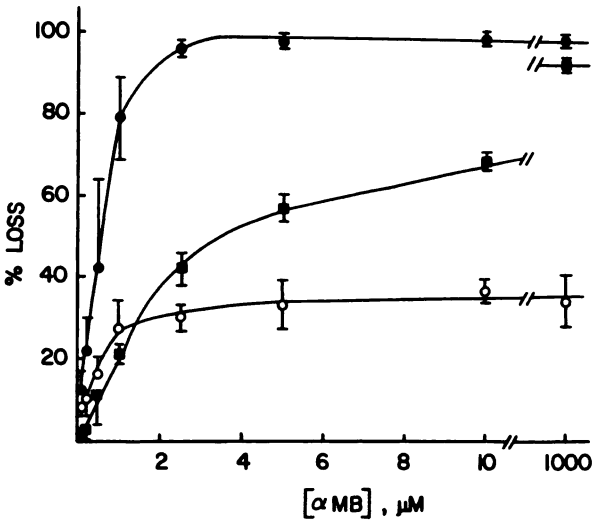

Fig. 4. Percentage loss of P-450 content and BND or 7-ERF activity in pulmonary microsomes (approximately 1 mg of protein/ml) from β-NF-treated rabbits after incubation with αMB and 1 mM NADPH for 45 min at 37°. One hundred per cent values for P-450 content (○), BND (●), and 7-ERF *O*-deethylase (■) activities were 0.43 ± 0.02 nmol/mg of microsomal protein, 8.0 ± 1.2 nmol/mg of microsomal protein/min, and 86 ± 8 pmol/mg of microsomal protein/min, respectively. Data plotted are means ± standard deviation, $N = 3$ where each N represents a pool of lungs from 9–12 rabbits.

TABLE 4

P-450 and heme losses in hepatic microsomes from PB-treated rats incubated with BBT or αMB[a]

Inhibitor	% Loss P-450[b]	Molar ratio, $\frac{\text{P-450 loss}}{\text{Heme loss}}$
BBT	42.0 ± 4.8	0.94 ± 0.01
αMB	46.0 ± 4.0	1.00 ± 0.10

[a] Rats were treated intraperitoneally once daily for 5 days with PB, 50 mg/kg. Hepatic microsomes were incubated with 0.5 mM BBT or αMB and 1 mM NADPH for 30 min.
[b] One hundred per cent values for specific contents of P-450 and heme were 1.16 ± 0.13 and 1.67 ± 0.17 nmol/mg of microsomal protein, respectively. Values are means ± standard deviation for $N = 3$ samples from individual livers.

450 was accompanied by a shift in wavelength for maximal absorption of the P-450 difference spectrum in the Soret region from 450–452 nm.

Isolation and characterization of hepatic pigment(s) formed after *in vivo* treatment of rats with BBT. Intraperitoneal administration of BBT to PB-treated rats caused the hepatic accumulation of an abnormal porphyrin pigment which was isolated on thin layer chromatographic plates and visualized as a green band fluorescing red upon irradiation with light from a short wavelength UV lamp. The physical properties of this pigment were compared with those of the porphyrin previously isolated after treatment of rats with ABT and characterized (7, 11) as a protoporphyrin IX derivative in which vicinal nitrogens are bridged with a benzene ring. These disubstituted porphyrins have physical properties that differentiate them from other alkylated porphyrins. The ABT- and BBT-derived porphyrins were identical in 1) chromatography on thin layer plates (two different solvent systems) with an R_f reflecting an unusually high polarity and, 2) resistance to chelation of Zn^{2+}. These porphyrins also had very similar visible spectra, characteristic of protoporphyrin IX bisubstituted on two vicinal nitrogen atoms (Fig. 5).

Effect of BBT and ABT on FCM activity in pulmonary microsomes from untreated rabbits. Incubation of pulmonary microsomes with ABT or BBT caused no diminution of the DMA *N*-oxidase activity from the control value of 10.1 ± 0.8 nmol of DMA N-oxide formed/mg of microsomal protein/min. Treatment with 10 μM BBT, 1 mM ABT, and 1 mM BBT resulted in activities of 10.4 ± 1.1, 11.2 ± 1.0, and 15.4 ± 0.1 nmol of DMA *N*-oxide formed/mg of microsomal protein/min, respectively (mean ± standard deviation for microsomes from three individual lungs).

Discussion

The microsomal P-450 monooxygenase system is responsible for the oxidation of numerous endogenous and exogenous compounds, and its terminal oxidase occurs as multiple isozymes which are under tissue-selective regulation (e.g. Ref. 5). Because P-450 isozymes have different, but overlapping, substrate specificities, methods have been developed to assess the contribution of individual P-450 isozymes to specific biotransformation reactions. Compounds that selectively inhibit or induce P-450 isozymes are routinely used in this regard as are polyclonal and monoclonal antibodies raised against highly purified P-450s. A complementary approach is the design of potent suicide substrates (24), also known as mechanism-based inhibitors (25). If such compounds could also be made tissue specific in effect (e.g., lung versus liver), they might be used *in vivo* to study the physiological role(s) of pulmonary P-450 as well as the contribution of specific pulmonary P-450 isozymes to whole body metabolism of xenobiotics toxic to lung. Other factors, such as pulmonary heme regulation, could also be investigated subsequent to destruction of P-450.

ABT was chosen as the parent structure for our analogues because it is a suicide inhibitor whose mechanism of action is known (7, 8, 11), it is relatively potent (6, 24), and it has a

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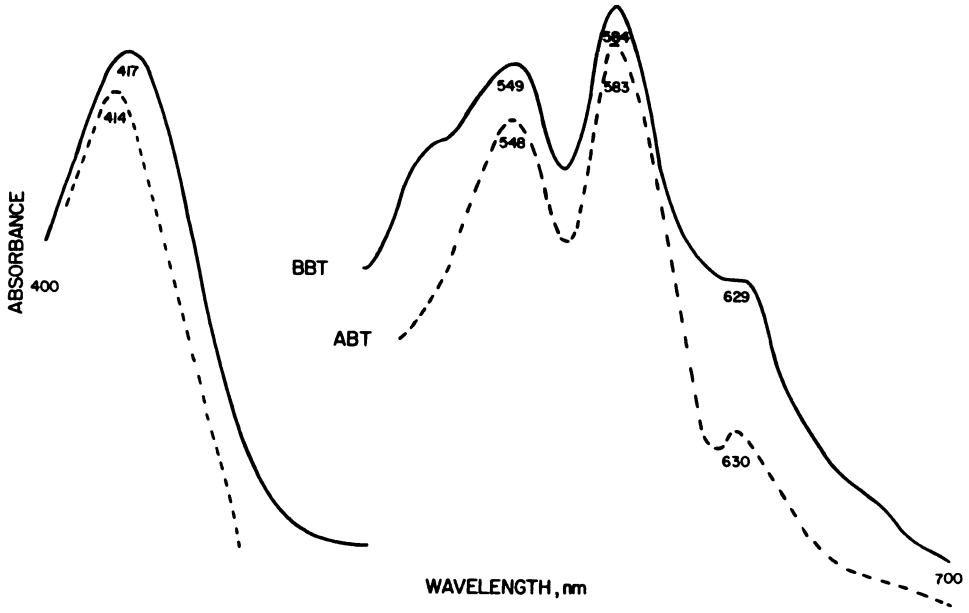

Fig. 5. Absorption spectrum of the modified hepatic porphyrin pigment isolated after intraperitoneal treatment of PB-treated rats with BBT (400 mg/kg). Male rats were treated intraperitoneally once daily with PB (50 mg/kg) for 5 days prior to administration of BBT. The spectrum of the modified hepatic porphyrin adduct isolated from rats treated with ABT (7) is shown for comparison.

structure that could readily be modified to derivatives with the major molecular features of benzphetamine, an isozyme-selective substrate of P-450. The rabbit pulmonary system was selected for our biological studies because it has two predominant P-450 isozymes, forms 2 and 5, that are under similar regulation in lung, but only form 2 has benzphetamine *N*-demethylase activity (3).

Consistent with previous work (6, 7–9), ABT was found to be a suicide inhibitor of P-450 with limited isozyme selectivity; virtually all of the P-450 in pulmonary microsomes was destroyed by 10 mM ABT (Fig. 1). *N*-Alkyl derivatives of ABT also inactivated pulmonary P-450, and they exhibited enhanced isozyme selectivity (Table 1). Two of the analogues, BBT and αMB, were also much more potent than ABT, exerting near-complete effects at 10 μM (Fig. 3) and 2.5 μM (Fig. 4), respectively. The partition ratios for these two *N*-alkyl analogues of ABT (a maximum value of 10–20 turnovers/enzyme destruction) are considerably higher than those measured for other suicide substrates of P-450, such as the classical inhibitor allylisopropylacetamide (230 turnovers/destruction; Ref. 26) or the cyclopropyl amines (60–520 turnovers/destruction; Ref. 27). MBT, although active, was less selective and less potent than BBT or αMB (Table 1). BuBT and BBT demonstrated nearly identical profiles of P-450 destruction in pulmonary (Table 1) and hepatic (data not shown) microsomes from untreated or β-NF-induced rabbits at inhibitor concentrations ranging from 10 μM to 1 mM. Thus, the *N*-benzyl group of BBT, which conferred selective suicidal activity against P-450 form 2 and structurally mimics the substrate benzphetamine (Fig. 1), was exchanged with an *N*-butyl group without apparent discrimination by P-450. This suggests that lipophilicity and molecular dimension near the site of oxidation, rather than aromaticity, are determinants of isozyme selectivity with these analogues.

Suicide inhibitors of P-450 are substrates for the monooxygenase system, and the efficiency of the catalysis of this metabolism (V_{max}/K_m) will determine their selectivity with individual isozymes of P-450. Consequently, the suicidal potency of an analogue (with the same mechanism of action as other compounds in the series) will increase with increasing V_{max} or decreasing K_m; i.e., isozyme selectivity will be a function of k_{cat}. We do not know what the relative contributions of V_{max} and K_m are to the selective destruction of P-450 form 2 by BBT and BuBT (versus ABT), but marked increases in lipophilicity almost certainly result in a decreased K_m in both cases.

Two of our analogues, BBT and BuBT, demonstrated remarkable selectivity between rabbit P-450 forms 2 and 5. Pulmonary form 2 was completely and irreversibly inactivated by a relatively low concentration (10 μM) of these inhibitors (Table 2); in marked contrast to ABT, no destruction of form 5 was observed at this and higher inhibitor concentrations. BuBT and BBT strongly inhibited form 6-catalyzed monooxygenase activity as well; this action was somewhat unexpected because this isozyme has no BND activity. The α-methylbenzyl derivative (αMB) of ABT was synthesized as a closer molecular mimic to benzphetamine in which steric hindrance about the amino group (the site of oxidation for the release of benzyne) is increased. Whereas BBT was almost equally effective against forms 2 and 6 (Fig. 3), αMB demonstrated a decided preference toward inactivation of form 2 over that of form 6 (Fig. 4).

The rabbit lung also contains significant amounts of FCM as demonstrated by its high *N*-oxidase activity (28, 29). However, neither the primary amine ABT nor the secondary amine BBT inactivated pulmonary microsomal FCM activity in the presence or absence of NADPH. In fact, 1 mM BBT caused a significant enhancement of FCM activity reminiscent of such "positive-effectors" as *n*-octylamine in rabbit lung microsomes (30). Consequently, the family of suicide inhibitors described here allows one to delineate the P-450-dependent monooxygenase system from the FCM, at least in rabbit lung. At 1 mM ABT, more than 70% of total pulmonary P-450 is inactivated without effect on FCM activity. In this respect these suicide inhibitors complement other methods currently available to distinguish between the two systems, including the selective inhibition of P-450-dependent activity of CO, by lipophilic primary alkylamines, or by specific antibodies to NADPH-P-450 reductase (31, 32).

We also determined the mechanism by which one of the more potent and selective *N*-alkylated ABT derivatives inactivates

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P-450. BBT was chosen as the compound and the experiments were performed with PB-induced rats where there is sufficient hepatic P-450 to make isolation of modified heme pigment(s) possible (7, 8, 11), as opposed to rabbit lung where this is not the case. The rat was also used because earlier experiments with ABT were performed in this species (11). The fact that a spectral complex was obtained after treatment with BBT (Fig. 5) that was very similar to the one obtained after treatment with ABT strongly suggests that oxidation occurs at the substituted amino group of BBT to release benzyne, which alkylates the porphyrin of P-450. The high isozyme selectivity of BBT (isozymes 2 and 6 versus 5) indicates that *N*-dealkylation to the nonspecific inhibitor ABT is not an important metabolic pathway. Oxidation by direct abstraction of electrons by nitrogen is postulated as a mechanism for *N*-dealkylation reactions catalyzed by P-450 (33). The *N*-alkylated ABT derivatives described here may allow a means of interceding (by substrate fragmentation and enzyme alkylation) in this process and may offer further evidence in support of this generalized mechanism.

There is one additional reason why we selected ABT and its derivatives as candidates for isozyme-selective suicide inhibitors of P-450. This is related to our goal of developing compounds that preferentially inactivate pulmonary P-450. There is a facilitative uptake system for lipophilic basic amines in lung (34). Although ABT is not a substrate for this uptake system due to its hydrophilic nature (6, 24), lipophilic analogues of ABT should be accumulated by lung. Preliminary *in vivo* experiments in our laboratory (35) suggest that this is true. Thus, 1 hr after intravenous administration, αMB (1 μmol/kg) and BBT (10 μmol/kg) inactivated more than 80% of pulmonary BND activity without affecting hepatic BND activity. These treatments also reduced the concentration of spectrally assayed P-450 in lung by approximately 30% without affecting hepatic P-450.

In this respect, Näslund and Halpert (36) recently reported that administration of chloramphenicol (300 μmol/kg) to rats inactivated the P-450 isozyme(s) that converts *n*-hexane to 2-hexanol in liver and lung. Interestingly, the isozyme that is selectively (i.e., versus other rat lung isozymes) destroyed by chloramphenicol in rat lung, P-450 PB-B, is the immunochemical homologue of rabbit form 2, which is selectively destroyed by αMB and BBT. There are two major differences between the ABT derivatives and chloramphenicol, however. First, αMB and BBT were specific for destruction of rabbit pulmonary versus hepatic form 2, whereas chloramphenicol showed no tissue preference; and second, the ABT analogues function by the formation of benzyne which binds P-450 heme, whereas metabolites of chloramphenicol bind to amino acid residues of P-450-PB close to the heme of the cytochrome, retarding electron transport by the monooxygenase (37).

In summary, we describe the successful design and synthesis of certain compounds that are potent and isozyme-selective suicide inhibitors of pulmonary P-450. Since oxidation on or about amine nitrogen is a common isozyme-selective reaction, the extension of ABT-based structural mimics to other isozyme-substrate pairs holds promise for the development of additional suicide inhibitors with high potency and selectivity, and with the potential to preferentially inactivate pulmonary versus hepatic P-450.

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