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1-Naphthylthiourea: A Mutagenic Rodenticide that Transforms Hamster Embryo Cells

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SUMMARY

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1-Naphthylthiourea is a rodenticide which produces pulmonary edema. It is mutagenic when metabolized by liver microsomal fractions from rats treated with either Aroclor 1254 or phenobarbital. It also transforms hamster embryo cells in an *in vitro* carcinogenesis bioassay.

INTRODUCTION

1-(1-Naphthyl)-2-thiourea, 1-naphthylthiourea, commonly called ANTU, is used as a rodenticide in many commercial products (1). It is a powerful lung toxin and is used for studying pulmonary edema (2). Recently, Boyd and Neal demonstrated that this compound is actively metabolized by the microsomal drug hydroxylation system in both liver and lung (3). A metabolite of 1-NTU[1] was shown to bind covalently to macromolecules of these tissues resulting in the subsequent destruction of the cytochrome P-450, an effect common for many thiono-sulfur-containing compounds (4–11).

Since 1-NTU has been shown to inhibit aromatic amine hydroxylation by the microsomal flavoprotein, amine oxidase (12–14), and cytochrome P-450-mediated reactions (3, 12), we attempted to use this compound as a selective inhibitor in the Ames *Salmonella*/mammalian-microsome mutagenicity test. Surprisingly, it was metabolized to a product that mutated the tester strain TA 1538 when added to assays containing post-mitochondrial supernatant fractions from livers of phenobarbital- or Aroclor 1254-treated rats. This report describes the results of these experiments and also those carried out with an *in vitro* hamster embryo cell transformation assay.

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[1] The abbreviations used are: 1-NTU, 1-naphthylthiourea; AC-, Aroclor 1254, a mixture of polychlorinated biphenyls; PB, phenobarbital; PB-S_9, 9000 × *g* supernatant from livers of phenobarbital-treated rats; AC-S_9, 9000 × *g* supernatant from livers of Aroclor 1254-treated rats; Ames assay, Ames Salmonella/mammalian-microsome mutagenicity test; HECT bioassay, hamster embryo cell transformation bioassay; DMSO, dimethylsulfoxide; R, rads; TLC, thin-layer chromatography; UV, ultra violet; IR, infra red; 1-NIT, 1-naphthylisothiocyanate; 2-NA, 2-naphthylamine.

MATERIALS AND METHODS

Reagents. The following chemicals used in these studies were obtained from commercial sources as follows: 1-naphthylthiourea (K & K), 1-naphthylisothiocyanate (P and B Research Chemicals), phenylthiourea (J. T. Baker, Co.), thiourea and sodium diethyldithiocarbamate (Eastman), sym.-diphenylthiourea, N-methylthiourea,

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thioacetamide and thiobenzamide (Aldrich), and NADPH from Sigma; phenobarbital from Sterling-Winthrop Aroclor 1254 from Analabs; 2-naphthylamine from the NCI Chemical Repository at IIT. All reagents were of the highest purity available commercially.

Animals and bacteria. The 9000 × *g* supernatant fractions were prepared from 25% homogenates of livers from male rats (Sprague-Dawley strain, 250 g or greater obtained from the NIH animal colony) induced by intraperitoneal injections with either PB (100 mg/kg/day for 3 days) or AC (500 mg/kg once 5 days prior to sacrifice). The *Salmonella typhimurium* tester strains TA 1535 and TA 1538 were supplied by Dr. B. N. Ames. The bacteria were grown in nutrient broth with shaking for 14 hr at 37°. Protein concentrations were determined by the method of Lowry *et al.* (15).

Purification and spectral analysis of 1-NTU. 1-NTU was purified by TLC on 1 mm silica gel plates (Analtech) developed with chloroform:ethylacetate (3:7). This procedure yielded two major areas on the chromatogram termed fraction A (3.5 mg) and fraction B (21 mg). Fraction A contained several bands with RFs > 0.7 which were visible under short λ UV light and did not react with pentacyanoaquoferriate reagent.[2] Fraction B had an RF = 0.6–0.7 and it appeared as a dark quenching band under long λ UV light. It stained blue with pentacyanoaquoferriate reagent. The fractions were eluted from the silica gel with acetone and dried under nitrogen. Rechromatography of fraction B in chloroform: hexane (9:1) showed the presence of only one band which was visible under UV light and which stained with pentacyanoaquoferriate reagent. IR spectroscopy of fraction B as a KBr pellet gave a spectrum identical to that previously reported for 1-NTU. No attempts were made to identify fraction A since it was composed of several bands on TLC.

[2] Pentacyanoaquoferriate reagent is a solution of potassium ferricyanide-sodium nitroprusside and is used to detect aromatic amines and other substituted amino group compounds. Its preparation and use are described in the CRC-Handbook of Chromatography.

The dried samples were dissolved in 10 ml ethanol. Ten μl aliquots of the starting 1-NTU solution (10 mg/ml) and fraction B, and 50 μl of fraction A were each diluted to 10 ml with ethanol and their spectra recorded on a Beckman DB-GT double-beam spectrophotometer. The solutions were then taken to dryness under reduced pressure. Based upon the calculated molar extinction coefficient at 282 nm for the starting 1-NTU (11,200 m^{-1} cm^{-1}), the final solution of fraction B was adjusted to give the same A_{282} reading as in the starting 1-NTU. Fraction A was diluted to the same volume as fraction B although its final concentration was ⅐ that of fraction B. These solutions were made up in DMSO and were subsequently used in the Ames and HECT assays.

Mutagenicity. The Ames assay with metabolic activation was carried out essentially as described by Ames *et al.* (16). A 0.2 ml aliquot of cells and 0.1 ml of the test compound dissolved in DMSO were sequentially added to 2 ml of the molten top agar with or without 0.5 ml of the S_9 reaction mixture (16). The S_9 from PB-treated animals was used without dilution but the AC-S_9 was diluted (1:1). These amounts were equivalent to 6 mg and 3 mg protein per test plate for AC-S_9 and PB-S_9-mediated reactions, respectively. Petri plates containing Vogel-Bonner E medium (17) were overlayed with the final assay mixture and incubated for 48 hr at 37°. Revertant colonies were counted manually. The results are presented as his^+ revertants (strain)/plate/mg S_9 protein. Each value represents the results of several experiments containing duplicate determinations.

Hamster embryo cell transformation (HECT) bioassay. Dulbecco's Modified Eagle Medium contained sodium pyruvate and low glucose content (Grand Island Biological Company, Grand Island, NY.). It was supplemented with 2 mM L-glutamine and 20% sterile filtered heat-inactivated fetal bovine serum (Rehatuin, F. S., Reheis Chemical Company, Phoenix, AZ). The medium contained no antibiotics. Embryos at 13 days of gestation from golden Syrian hamsters of the outbred, systematically randomized ELA/ENG strain (Engle's Lab-

oratory Animals, Inc., Farmersburg, IN) served as the source of primary embryo cells used as target or feeder cells. The bioassay used in this study was run without added metabolic activation and is described in detail elsewhere (18). Briefly, an ampoule of frozen feeder cells was reconstituted and cultured at 37° to 75–80% confluence. The feeder cell cultures were then X-irradiated with 5000R and seeded into 50 mm plastic petri dishes at a density of 6×10^4 cells per dish in 2 ml of culture medium. Twenty-four hours later, similarly reconstituted target cell cultures grown to 80–90% confluence were treated with trypsin to detach them from the petri dishes and approximately 500 cells were seeded in 2 ml of culture medium onto the feeder cells. On the next day, graded doses of test chemicals, prepared double strength in culture medium, were added to the 4 ml of culture medium in the dishes to obtain the desired final concentrations. Control cultures were treated with medium alone, or culture medium containing 0.2% DMSO, the solvent used in this bioassay. The cultures were incubated for 8 days at 37° in 10% CO_2 and air. They were then washed twice with physiological saline solution, rinsed once with methanol to remove residual test compounds (19), fixed with methanol and stained with Giemsa. The stained dishes were examined with an Olympus model JM zoom stereomicroscope (7–40 × magnification) to identify normal and transformed colonies.

RESULTS

1-NTU was metabolized by post-mitochondrial supernatant fractions of rat liver homogenates to mutagenic products. The results obtained were independent of the source of the activating enzyme(s), i.e., by liver S_9-fractions from either PB- or AC-treated rats. 1-NTU was not mutagenic toward TA 1538 or TA 1535 in the absence of either S_9-fraction.

When the results of six different experiments were compiled in order to compare the ability of these two enzyme preparations to metabolize 1-NTU to mutagenic metabolites, essentially no statistical difference was observed in the dose response curves. These combined results ± SD are shown in Table 1.

TABLE 1

Comparison of AC- and PB-S_9's to metabolize 1-NTU to mutagenic metabolite

Ames assays using the pour plate procedure were performed as described in METHODS. The protein concentrations of the S_9-fractions were 3 and 6 mg protein/assay for the AC- and PB-S_9 fractions, respectively. Background levels were 19 ± 7 His^+ revertants per plate in the absence of added S_9-fractions.

μg 1-NTU added/plate	His^+ revertants (TA 1538)/plate/mg S_9 ± SD (n = 8)	
	AC-S_9	PB-S_9
0.0	12 ± 3	5 ± 1
1.0	16 ± 3	8 ± 5
2.5	25 ± 5	29 ± 10
5.0	50 ± 20	61 ± 16
10.0	80 ± 13*	131 ± 27*
20.0	164 ± 23*	238 ± 20*
25.0	208 ± 45	221 ± 44
50.0	389 ± 71	368 ± 29
100.0	591 ± 67*	398 ± 78*

* Results are significantly different ($p < 0.01$) using paired Student's *t*-test.

Two major fractions, A and B, were obtained from TLC of 1-NTU on silica gel as described in MATERIALS AND METHODS. Since fraction B was essentially identical to 1-NTU with respect to its IR and UV-spectra, and represented the majority of material on TLC, we inferred that fraction B was 1-NTU. Fraction A was a mixture of several contaminating bands on TLC and was not further characterized. When these two fractions were tested for their possible metabolism to mutagenic products in the Ames Assay (Fig. 1), fraction A gave very low results with both PB- and AC-S_9's, whereas fraction B gave essentially the same response as the commercially-available 1-NTU. Neither TLC fraction was mutagenic in the absence of added S_9's. Even though the values obtained for fraction A were between 40 and 100 revertants/plate/mg S_9, they were still several-fold above background values for this assay, i.e., 10 and 25 revertants/plate/mg S_9 for PB- and AC-S_9's, respectively. The identities of these contaminants are unknown.

Since the 1-NTU was mutagenic in the Ames assay, it was tested for its carcino-

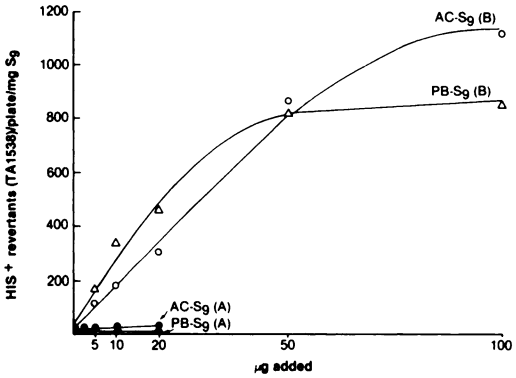

FIG. 1. *Dose response curve for S. typhimurium TA 1538 toward TLC fractions obtained from 1-NTU and metabolized by AC- or PB-S_9's*

The TLC separation was achieved as described in MATERIALS AND METHODS. The mutagenicity data were obtained using the normal pour plate procedure. Each point is the average of duplicate determinations. Fraction A: with PB-S_9 (▲——▲); with AC-S_9 (●——●); Fraction B: with PB-S_9 (△——△), with AC-S_9 (○——○).

genic potential using the improved HECT bioassay developed by Pienta *et al.* (18). A screening test using log-fold doses (0.1–100 µg/ml) indicated positive results in the range of 1–10 µg/ml. Assays were repeated within the narrower dose range using the commercially-available unfractionated 1-NTU and the two fractions that were isolated by TLC. The results of these experiments are shown in Table 2. It is evident that the 1-NTU gave a good response within the range tested (1.6–25 µg/ml). The two individual TLC fractions were tested at concentrations which were proportional to that present in the original 1-NTU sample. The individual TLC fractions transformed the hamster embryo cells only at the highest dose tested. It was surprising that both TLC fractions were positive in the HECT assay, especially in light of the low mutation levels obtained with fraction A in the Ames assay.

From the results of Boyd and Neal (3) and Fok (12) it is obvious that 1-NTU must be metabolized to exert its effects. Even though they made no attempt to identify the metabolite(s) of 1-NTU, they demonstrated that metabolism resulted in the binding of a sulfur-containing derivative to TCA-precipitable materials. Recently, Lee and Neal (21) reported that 1-NTU was metabolized to 1-naphthylurea and atomic sulfur.

Since 1-NTU has been reported to be metabolized by the amine oxidase (12) and yet it also destroys cytochrome P-450 and interferes with other cytochrome P-450 mediated reactions (3), its metabolism could also be catalyzed by cytochrome P-450. Since there are different forms of cytochrome P-450 induced by treatment with either PB or AC, then there should be differences in reactions containing either PB- or AC-S_9-fractions. However, the substrate dose response curves for 1-NTU in the Ames assay appear to be essentially the same irrespective of the S_9-fractions used (Table 1).

TABLE 2

Transformation of golden syrian hamster embryo cells[a]

The hamster embryo cell transformation assay was performed as described in METHODS.

Test compound[b]	µg/ml	# of dishes	Transformed colonies/surviving colonies
Tissue culture medium	—	8	0/792
0.2% DMSO[c]	—	7	0/693
3-Methylcholanthrene[d]	0.5	8	3/480
	1.0	8	3/480
	1.0	9	4/545
1-(1-Naphthyl)-2-thiourea	1.56	9	1/755
	3.12	9	3/894
	6.25	9	3/918
	12.5	9	4/894
	25	9	4/827
TLC-fraction A	0.25	9	0/600
	0.50	9	0/519
	1.00	9	0/707
	1.56	9	0/725
	3.12	9	3/744
TLC-fraction B	1.56	9	0/845
	3.12	9	0/800
	6.25	9	0/895
	12.5	9	0/788
	25	9	6/671

[a] Hamster embryo cell culture #76-582.

[b] Tested as coded samples. The identities of the compounds were not revealed until the results were tabulated.

[c] Solvent control.

[d] Positive carcinogen control.

TABLE 3

Comparison of mutagenicity of 2-naphthylamine and 1-naphthylthiourea using two tester strains

Performed as described in the text using the normal pour plate procedure.

μg added/ plate	his$^+$ revertants (TA 1538)/plate						his$^+$ revertants (TA 1535)/plate					
	NO-S_9		AC-S_9		PB-S_9		NO-S_9		AC-S_9		PB-S_9	
	2-NA	1-NTU	2-NA	1-NTU	2-NA	1-NTU	2-NA	1-NTU	2-NA	1-NTU	2-NA	1-NTU
0.0	18	22	29	26	23	22	30	30	17	19	22	19
1.0	27	27	44	53	24	25	35	11	80	20	65	19
2.5	12	25	45	74	27	52	29	11	265	14	98	15
5.0	12	21	46	142	36	243	18	12	602	14	242	19
10.0	18	23	81	232	55	432	34	8	1080	20	604	19
20.0	20	—[a]	82	—	54	—	39	—	884	—	1312	—
25.0	—	23	—	538	—	970	—	11	—	26	—	19
50.0	—	23	—	1248	—	1548	—	14	—	23	—	21
100.0	15	21	100	1868	—	1394	—	15	—	19	—	19
1000.0	15	—	120	—	—	—	—	—	—	—	—	—

[a] — = Not done.

In an attempt to determine if the mutagenicity of 1-NTU as observed in the Ames assay was the result of its thiourea linkage, we tested several other thiourea derivatives and analogues using two tester strains, TA 1535 and TA 1538, within the same dose response range that gave positive results for 1-NTU, namely, 1–100 μg/plate. These compounds included: thiourea, N-methylthiourea, phenylthiourea, sym.-diphenylthiourea, thioacetamide, thiobenzamide, sodium diethyldithiocarbamate and 1-naphthylisothiocyanate.

Only 1-NTU was metabolized to mutagenic metabolites. Since only the naphthyl derivative was positive, one possible explanation for this result was that it was contaminated with 2-NA. There was no indication of the presence of an aromatic amine in 1-NTU based upon its TLC mobility or its lack of reactivity with N,N-dimethylbenzaldehyde. Negative results were obtained in the Ames assay under conditions where a 1% level of contamination by 2-NA (80 rev/μg 2-NA with TA 1535) would have been detected (Table 3). Whatever the contaminants of 1-NTU are, they contribute very little to its metabolism to mutagenic products (Fig. 1).

DISCUSSION

The data presented here clearly demonstrate that 1-NTU can be metabolized to mutagenic metabolites by liver S_9-fractions from either AC- or PB-treated rats and also that it and some contaminant(s) in the commercially-available compounds can transform hamster embryo cells in an *in vitro* carcinogenesis assay.

The nature of all the metabolite(s) of 1-NTU is unknown. Besides the formation of free sulfur and 1-naphthylurea (20), Boyd and Neal (3) have demonstrated a definite level of nonspecific binding of either ^{14}C- or ^{35}S-labelled 1-NTU *in vitro* which is increased in the presence of NADPH. They observed that very little of the carbonyl carbon is bound relative to the thionosulfur (approximately 6-fold less ^{14}C than ^{35}S) thereby indicating release and binding of the sulfur atom from 1-NTU. No binding occurred with the oxygen analogue.

As reported by McCann *et al.* (21), we did not observe any metabolism of thiourea or thioacetamide to mutagenic metabolites even though these compounds are known carcinogens. In this same respect, the noncarcinogenic but hepatotoxic 1-NIT, the commercial precursor of 1-NTU, was not metabolized to mutagenic products in the Ames assay nor was it positive in the HECT assay (18). Only recently 1-NIT has been shown to bind irreversibly to rat liver microsomes when administered *in vivo* or metabolized *in vitro* (22). As with 1-NTU, these workers implicated an enzymatic mechanism for binding of 1-NIT but failed to show the nature of the metabolite. With

this compound, however, the ring-labeled substrate was bound to the same degree as the sulfur-labeled form. If the metabolism of 1-NTU is similar to that of 1-NIT, then the naphthyl group of both are probably incorporated at the site of binding. No attempts have been made to demonstrate binding to nucleic acids with these and other sulfur-containing compounds.

1-NTU has been suspected as a potential human carcinogen primarily because commercial preparations may be contaminated with traces of the 1- and 2-naphthylamines (23). Our studies seem to rule out any significant naphthylamine contamination of 1-NTU. Since 1-NTU is easily metabolized to a form which binds to macromolecules (3), its metabolites are mutagenic to bacteria, and it transforms mammalian cells, extreme care should be exercised by investigators in the handling of this compound.

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