STRUCTURAL REQUIREMENTS FOR MUTAGENIC ACTIVITY OF 2-ACYLAMINOFLUORENES IN THE SALMONELLA TEST SYSTEM

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SUMMARY: Twenty-nine 2-acylaminofluorenes were compared in the Salmonella typhymurium TA-1538 test system to determine the effect of structural changes on mutagenic activity. All the 2-acylaminofluorenes require the S-9 liver fraction for mutagenic activity. Mutagenic activity was highest in carbamate esters, ureas, α-haloacetylamides, and straight chain acylamides. Branching on the α-carbon, or addition of unsaturation reduced mutagenic activity. Pretreatment of C57BL/6N mice with 3-methylcholanthrene increased (6-20 fold) the mutagenic activity of all the analogs in the S-9 liver fraction. No increase in mutagenic activity is observed in S-9 liver fraction from 3-methylcholanthrene treated DBA/2N mice, indicating that mutagenic activity correlates with inducible aromatic hydrocarbon (benzo[a]pyrene) hydroxylase activity and N-hydroxylase activity. α-Naphthoflavone, an inhibitor of N-hydroxylation of 2-acetylaminofluorene in vitro, inhibits mutagenic activity of these analogs indicating that N-hydroxylation is the first step in activation of N-acylaminofluorenes into mutagens.

INTRODUCTION

2-AAF[1] is a widely studied carcinogen (1-3) and a potent mutagen in the Ames assay (4). Studies on the metabolism and carcinogenicity of 2-AAF have provided much of our current understanding of chemical carcinogenesis of aromatic amine and amides (1-3). N-hydroxylation of 2-AAF, by rodent liver enzymes both in vivo and in vitro, converts it to the more potent carcinogen N-OH-2-AAF (4-7), which also is a potent mutagen. Although N-hydroxylation is considered to be necessary in the initiation of cancer by 2-AAF, several findings indicate that the ultimate carcinogenic species may be a further metabolite(s) of N-OH-2-AAF rather than the compound itself (8,9).

N-OH-2-AAF is a substrate for variety of liver enzyme systems, which convert it

[1]Abbreviations: 2-AAF = 2-acetylaminofluorene; 2-AF = 2-aminofluorene; N-OH-2-AAF = N-hydroxy-2-acetylaminofluorene; 3-MC = 3-methylcholanthrene; α-NF = α-naphthoflavone.

to other electrophilic species. These enzyme systems include sulfotransferase, deacetylase, trans-acetylase and UDP-glucuronyltransferase (9-13). A chemical or enzymatic one-electron oxidation system, which converts it to nitroxide free radical which can then dismutate to the potent carcinogenic electrophiles N-acetoxy-2-AAF and 2-nitrosofluorene may also be important (14,15). The relative importance of these enzyme systems in converting N-OH-2-AAF to the ultimate carcinogenic species is not clear.

In mutagenesis testing, 2-AAF is a potent mutagen only in the presence of S-9 liver fraction (16). N-OH-2-AAF, while being a mutagen in the absence of the S-9 liver fraction, is more potent in its presence, suggesting further metabolism activates this compound to a more potent mutagen (16). The fact that metabolites, which could result from some of these processes are highly potent mutagens, even in the absence of S-9 fraction, e.g. N-hydroxy-2-aminofluorene (N-OH-2-AF) and 2-nitrosofluorene (17), also indicates further metabolism could be important in mutagenesis.

In this communication, we report our initial experiments to study the relationshi between mutagenic activity and structural changes in 2-acylaminofluorenes, as a step to obtain more information about structural requirements for the enzymic processes related to mutagenesis and carcinogenesis.

MATERIALS AND METHODS

2-Acylaminofluorenes. Acylation of 2-AF (Aldrich Chemical Co.) was accomplished using the appropriate acyl halides, anhydride or isocyanate. All were obtained from commercial sources except formic acetic anhydride which was prepared by the method of Stevens and Van Es (18). Reactions were run in $CHCl_3$ (5 mmol of 2-AF/15 ml $CHCl_3$) at C in the presence of one equivalent of pyridine, or in pyridine. The acyl halide was added in $CHCl_3$ over 1-3 minutes. Reaction progress was monitored by TLC, silica gel GF, 250 μ plates, developed using $CHCl_3$/EtOAc 80:20, Rf for 2-AF = 0.75 (acylated fluorenes usually had Rf values of 0.85-0.90). Upon disappearance of 2-AF by TLC, the solvent was evaporated, and product crystallized from EtOH-H_2O (charcoal). Reactions run in pyridine were terminated with ice-water and HCl. The propiolyl (HC≡C-CO-AF) analog was prepared using dicyclohexylcarbodiimide and propiolic acid in THF (0°) followed by addition of 2-AF. Urea nitrate was used for preparation of N-2-fluorenylurea. N-2-fluorenyl-N',N'-dimethylurea was prepared by allowing dimethylamine to react with the phenyl carbamate ester in EtOH, 45°, 30 min. All compounds gave satisfactory mass spectral fragmentation patterns, typical of 2-acylaminofluorenes (19).

Mutagenesis. Mutagenesis assays were performed as previously described (20,21). Test compounds were routinely added to 2 ml of top agar in 0.1 ml DMSO, except a high

concentrations when up to 0.5 ml DMSO was used. Bacterial tester strain TA-1538 ($2-3 \times 10^9$ bacteria/ml), 0.15 ml, was used. Cofactor solutions, 0.5 ml, contained the S-9 liver fraction, 2.0 mg, prepared from C57BL/6N or DBA/2N mice (control or 3-MC pretreated), 8 µmoles of $MgCl_2$, 33 µmoles KCl, 5 µmoles of G-6-P, 4 µmoles of NADP and 100 µmoles of sodium phosphate, per ml. In assays where α-NF was used as an inhibitor, it was added (in 0.1 ml of DMSO) to bacteria, cofactors and top agar, and mixed. The test compound was added last. Colonies (histidine revertants) were counted after a 2-day incubation at 37°. In control experiments on nutrient agar, no toxicity (reduction in bacterial growth) was observed for compounds at concentrations below 10 µg/plate, or in the range where mutagenic activity was obtained.

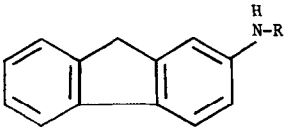
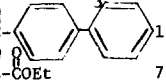
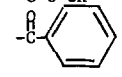


TABLE 1. Relative Mutagenic Activity of 2-Acylaminofluorenes

All compounds were tested as described in Materials and Methods section using S-9 fractions from C57B/6N mice pretreated with 3-MC (80 mg/kg) 40 hrs before sacrifice. Background revertants (DMSO in presence of cofactors, 50 colonies) are subtracted. Compounds were routinely tested at 0.1-10 µg of compound per plate, and up to 500 µg when few or no colonies were observed at lower concentrations.

Active (more than 80 colonies/nmol)		Less Active (10-75 colonies/nmol)		Weakly Active (less than 10 colonies/nmol)	
A. Amide Analogs					
-C(=O)-H	80				
-C(=O)-CH_3 (2-AAF)	141				
-C(=O)-CH_2CH_3	122				
-C(=O)-$CH_2CH_2CH_3$	115	-C(=O)-$CH(CH_3)_2$	44	-C(=O)-$C(CH_3)_3$	3
-C(=O)-$CH_2CH_2CH_2CH_3$	141			-C(=O)-$CH_2C(CH_3)_3$	<1
-C(=O)- 	169	-C(=O)- 	39	-C(=O)-C(CH_3)=CH_2	4
		-C(=O)-$CH=CH_2$	54	-C(=O)-$CH(CH_3)CH=CH_2$	5
-C(=O)-CH_2Cl	122	-C(=O)-C≡CH	21	-C(=O)- 	1
-C(=O)-$CHCl_2$	159	-C(=O)- 	26	-C(=O)-C(=O)Et	7
-C(=O)-CCl_3	81				
-C(=O)-CH_2Br	211				
-C(=O)-CH_2OCH_3	104				
-S(=O)(=O)-CH_3	188				
B. Ureas and Carbamate Analogs					
-C(=O)-NH_2	176	-C(=O)-$NHCH_3$	67	-C(=O)-NH- 	<1
		-C(=O)-$N(CH_3)_2$	11		
-C(=O)-OMe	456				
-C(=O)-O- 	338				

RESULTS AND DISCUSSION

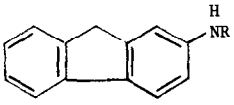
The relative mutagenic activity, expressed as revertants/nmole, of the 2-acylaminofluorenes are shown in Table 1. All compounds required the presence of S-9 liver fraction for mutagenic activity. A greater than 400 fold range of activity was seen. The most potent mutagens were the straight chain alkyl or α-X-substituted acyl derivatives; e.g., $CH_3CH_2\overset{O}{\overset{\|}{C}}-$, $CH_3CH_2CH_2\overset{O}{\overset{\|}{C}}-$, $ClCH_2\overset{O}{\overset{\|}{C}}-$, $BrCH_2\overset{O}{\overset{\|}{C}}-$, etc.; carbamate esters, e.g., $H_3CO\overset{O}{\overset{\|}{C}}-$, $PhO\overset{O}{\overset{\|}{C}}-$; and urea analogs; e.g., $H_2N\overset{O}{\overset{\|}{C}}$. Alkyl chain compounds retained good mutagenic activity unless branched, or when unsaturation was added. Branching greatly decreases mutagenic activity; e.g., t-butylacetyl and trimethylacetyl are among the least active. Increasing substitution on the nitrogen of the urea analogs also decreases mutagenic activity, $PhHN\overset{O}{\overset{\|}{C}}-$ < $(CH_3)_2N\overset{O}{\overset{\|}{C}}-$ < $CH_3HN\overset{O}{\overset{\|}{C}}-$ < $H_2N\overset{O}{\overset{\|}{C}}-$. Among saturated and unsaturated compounds, olefinic analogs were consistently less potent than the corresponding saturated analogs; e.g., acrylyl < propionyl, crotonyl < n-butyryl, and methacrylyl < iso-butyryl. The single exception to effects of branching seems to be cyclobutylcarbonyl. Other cycloalkylcarbonyl compounds have not been tested. α-Haloacetyl, α-multihalosubstituted-acetyl, and α-methoxyacetyl-2-AF all showed high mutagenic activity. The most surprising results are those in part B of Table 1 showing that carbamate and urea derivatives are more active mutagens than 2-AAF in this system, indicating that analogs other than acyl amides are readily converted to potent mutagens.

To answer the question whether N-hydroxylation is required for mutagenesis of these compounds, α-NF, a potent inhibitor of cytochrome P_1-450 dependent N-hydroxylation of 2-AAF (22) was added to the mutagenesis assays. Results with several analogs are shown in Table 2. Since α-NF has been shown to block N-hydroxylation, and had no toxic effect on the bacteria, it is inferred that N-hydroxylation is required for mutagenesis.

It has previously been shown that metabolic activation of 2-AAF into a mutagen is associated with the genetically mediated difference in aromatic hydrocarbon-inducible hepatic aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity, which can also be related to N-hydroxylation of 2-AAF (23). The aryl hydrocarbon hydroxylase responsive

TABLE 2. Effect of α-NF on Mutagenicity of Selected 2-Acylaminofluorenes

Compounds were tested as described in Materials and Methods section using the S-9 liver fraction from C57B/6N mice pretreated with 3-MC (80 mg/kg) 40 hours before sacrifice. Background revertants (DMSO in the presence of cofactors, 50 colonies) are subtracted.

		Per Cent Reduction	
	colonies/μg on plate	.005mM α-NF	0.05mM α-NF
$-\overset{O}{\overset{\|}{C}}-CH_3$ (2-AAF)	1250/2	40	95
$-\overset{O}{\overset{\|}{C}}-\underset{CH_3}{\underset{\|}{CH}}-CH_3$	1500/10	70	>98
$-\overset{O}{\overset{\|}{C}}-O-CH_3$	1850/1	35	95
$-\overset{O}{\overset{\|}{C}}-CH_2Br$	1320/2	51	>98
$-\overset{O}{\overset{\|}{C}}-CH_2Cl$	875/2	61	>98
$-\overset{O}{\overset{\|}{C}}-NH_2$	1525/2	73	>98
$-\overset{O}{\overset{\|}{C}}-NHCH_3$	1350/5	84	>98
$-\overset{O}{\overset{\|}{\underset{O}{\underset{\|}{S}}}}-CH_3$	1400/2	86	>98
$-\overset{O}{\overset{\|}{C}}-CH=CH_2$	635/5	67	>98

mouse, C57BL/6N, shows an approximately 20 fold increase in mutagenic activity of 2-AAF when comparing liver fractions from 3-MC pretreated and control animals (23). Parallel experiments with S-9 fractions from nonresponsive DBA/2N mice showed no increase in mutagenicity of 2-AAF, or of N-hydroxylation in vitro (21,23). The analogs showed increases of 6-20 fold in mutagenic activity when comparing S-9 liver fraction from 3-MC pretreated C57B/6N mice to controls, and no increases when similar fractions from DBA/2N mice were used, suggesting that mutagenic activity dependents upon N-hydroxylation.

Our results clearly show that N-hydroxylation is required for activation of these 2-acylaminofluorenes to potent mutagens. The differences in mutagenic activity among the analogs could reflect differences in the rate of cytochrome P_1-450 dependent N-hydroxylation, but may also reflect the ability of the hydroxamic acids so formed

to undergo subsequent activation steps (e.g. deacylation) which have been shown to be important in 2-AAF caused mutagenesis (23). Studies on several aspects of this problem are in progress.

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