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The in vivo and in vitro genotoxicity of aromatic amines in relationship to the genotoxicity of benzidine

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Summary

Benzidine and 12 related aromatic amines have been studied for the effects of substituent groups and π orbital conjugation on their genotoxicity as measured by their mutagenicity in vitro with Salmonella and by chromosomal aberrations (CA) in vivo in the bone-marrow cells of mice. The in vitro studies indicated increases in mutagenicity with increases in the electron withdrawing ability of *para'* substituents. Mutagenicity also increases with increased conjugation as shown by the degree of planarity of the biphenyl compounds and by comparing the mutagenicities of biphenyl amines to stilbenes as well as to ethylene bridged diphenyl compounds. The relative in vitro mutagenicity results were not predictive of relative in vivo CA results. The 3 most genotoxic compounds in vivo were the conjugated amines without substituents in the *para'* position. The CA values for 4-aminostilbene were exceptionally high. These in vivo results indicate increased genotoxicity for benzidine analogs without substitution in the *para'* position.

In a previous study of a series of commercially available aromatic amines related to benzidine (Messerly et al., 1987), we developed some structure–mutagenicity relationships based upon in vitro results from Ames testing with Salmonella (Maron and Ames, 1983). In the present study, we are expanding that study to include both in vivo and in vitro structure–genotoxicity relationships. Model aminobiphenyl and aminostilbene compounds have been synthesized and, together with key compounds from the previous study, have been evaluated for in vivo chromosomal aberrations (CA) in the bone marrow of mice for comparison to their Ames test results.

Because of the recognition of benzidine as a human carcinogen (Zavon et al., 1973; Haley, 1975) and with the resulting limitations that exist on its manufacture as well as use (US Federal Register, 1974), there has been a need for suitable substitutes especially for use in the dye and polymer industries. Structure–genotoxicity relationships that could be applied to such compounds should be helpful in reaching risk-assess-

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ment decisions concerning their potential hazards.

Planarity and an activated electrophilic site were indicated to be important factors contributing to the mutagenicity of benzidine analogs in TA98 with S9 activation (Messerly et al., 1987). It is the purpose of the present investigation to test further the effect of these factors in Salmonella strains TA98 and TA100. There is the reported need to supplement the ability of a bacterial test to detect point mutations with a short-term procedure that responds to strand breaks in vivo (Natarajan and Obe, 1986; Shelby, 1988). Therefore, we have also included in this paper a comparison of the genotoxicity of our test compounds in vivo as measured by CA in the bone-marrow cells of mice. Towards these ends we have tested a limited series of 4-aminobiphenyls, 4-aminostilbenes and related compounds with various substituents in the 4′-position and determined the extent to which their in vitro Salmonella results are predictive of their in vivo CA results.

Materials and methods

Animals

Male CD1 mice 10–12 weeks old (~ 30 g) and Sprague–Dawley male rats (~ 200 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Mice were kept 4 per cage and rats 3 per cage with wood chip bedding, were fed chow (Purina) and water ad libitum and were housed 4–5 days prior to the start of the experiment.

Chemicals

4-Aminobiphenyl, 4,4′-ethylenebis(aniline), 2-aminofluorene, 2,7-diaminofluorene and 7,12-dimethylbenz[*a*]anthracene (DMBA) were purchased from Aldrich Chemical Company (Milwaukee). 4,4′-Diaminobiphenyl (benzidine) was purchased from Harleco (Philadelphia). 4-Amino-4′-chloro-, 4-amino-4′-nitro- and 4-amino-2′-methylbiphenyl as well as 4-amino-, 4,4′-diamino-, 4-amino-4′-chloro- and 4-amino-4′-nitrostilbene were synthesized from their corresponding nitro precursors by a Zinin reduction (Porter, 1973) with an excess amount of NaHS in refluxing methanol/water. Reactions for the formation of 4-amino-4′-nitrobiphenyl as well as 4-amino-4′-nitrostilbene were closely monitored to minimize over-reduction while the other amino compounds were produced by prolonged refluxing to achieve high yields. 4-(2-Phenylethyl)aniline was obtained in nearly quantitative yield after silica gel chromatography by hydrogenation (4 atm) of 4-nitrostilbene in ethyl acetate with Pd-C (10%) as catalyst.

In regards to the required nitro intermediates, 4-chloro-4′-nitrobiphenyl was synthesized by the procedure of Beaven et al. (1961). 4,4′-Dinitrobiphenyl was obtained from COC Company Inc. (Columbia, SC) and 2-methyl-4′-nitrobiphenyl was synthesized by the reaction of the diazonium salt of *p*-nitroaniline with toluene by the procedure of France et al. (1939). The nitrostilbenes were obtained by the Wittig reaction with (4-nitrobenzyl)triphenylphosphonium bromide and the required commercially available 4-substituted benzaldehydes (Aldrich). The method used was modified from that of Geerts and Martin (1960) as illustrated in the following synthesis of 4,4′-dinitrostilbene.

p-Nitrobenzyl bromide (2.0 g, 9.2 mmoles) and triphenylphosphine (2.6 g, 9.9 mmoles) in benzene (8.0 ml) were refluxed for 36 h. The phosphonium intermediate was obtained in 99% yield by filtering the reaction mixture at room temperature, washing the precipitate thoroughly with benzene and drying under vacuum. To a mixture of *p*-nitrobenzaldehyde (1.0 g, 6.6 mmoles) and (4-nitrobenzyl)triphenylphosphonium bromide (4.5 g, 10 mmoles) in methanol, (20 ml) sodium methoxide (0.544 g, 10 mmoles) was added in small portions. The resulting suspension was stirred for 1 h, filtered and the precipitate was washed with methanol to yield a mixture of E and Z-stilbene isomers. This mixture together with iodine (0.2 g) was dissolved in 240 ml of ethyl acetate and heated at reflux for 12 h to isomerize the Z to the E isomer. The yellow crystals, obtained by filtration at room temperature together with those formed when the mother liquor was condensed to 100 ml, were washed with methanol and dried under vacuum to yield 1.56 g (87%) of the desired product: m.p. 298–300°C [lit. (Geerts and Martin, 1960), 303–304°C].

The synthesized test compounds were purified

by silica gel chromatography and characterized by melting point and NMR. Table 1 summarizes the yields of the test compounds as obtained from their corresponding nitro precursors, their melting points and NMR data. All test compounds at 0.02 μmoles showed a single spot under UV light with TLC using prescored silica gel GHLF uniplates (Analtech Inc., Newark, DE) in hexane/ethyl acetate solvent systems.

Mutagenicity

Mutagenicity was determined with Salmonella strains TA98 and TA100 by the standard plate incorporation procedure with and without Aroclor 1254 induced rat-liver S9 (10%) activation as outlined by Maron and Ames (1983). Each concentration in DMSO indicated in Table 2 was run at least in triplicate and the dose–response relationship confirmed on a second day. Plates were

TABLE 1

YIELDS, MELTING POINTS, AND PROTON NMR DATA[a] OF THE SYNTHESIZED TEST COMPOUNDS

Compounds	Yields (%)	M.p., °C (lit. m.p.)	Proton NMR (300 MHz, $CDCl_3$)
4-Amino-4'-chlorobiphenyl	98	129–131 (133–134)[b]	3.75 (2H, br s), 6.748 (2H, d, J = 8.4 Hz) 7.346 (2H, d, J = 8.5 Hz), 7.369 (2H, d, J = 8.4 Hz), 7.449 (2H, d, J = 8.5 Hz).
4-Amino-4'-nitrobiphenyl	55	203–204 (203–204)[b]	3.891 (2H, br s), 6.779 (2H, d, J = 8.7 Hz), 7.473 (2H, d, J = 8.7 Hz). 7.664 (2H, d, J = 9.0 Hz), 8.243 (2H, d, J = 9.0 Hz).
4-Aminostilbene	98	147–149 (148–149)[c]	3.749 (2H, br s), 6.680 (2H, d, J = 8.6 Hz), 6.92 (1H, d, J = 16.3 Hz), 7.02 (1H, d, J = 16.3 Hz), 7.208 (1H, tt, J = 8.0, 1.5 Hz), 7.332 (2H, t, J = 8.0 Hz), 7.342 (2H, d, J = 8.6 Hz), 7.473 (2H, dd, J = 8.0, 1.0 Hz).
4,4'-Diaminostilbene	99	230–231 (231)[d]	3.695 (4H, br s), 6.663 (4H, d, J = 8.5 Hz), 6.839 (2H), 7.294 (4H, d, J = 8.5 Hz).
4-Amino-4'-chlorostilbene	95	198–200 (185–187.5)[c]	3.761 (2H, br s), 6.673 (2H, d, J = 8.5 Hz), 6.851 (1H, d, J = 16.4 Hz), 6.986 (1H, d, J = 16.4 Hz), 7.280 (2H, d, J = 8.6 Hz), 7.319 (2H, d, J = 8.5 Hz), 7.385 (2H, d, J = 8.6 Hz).
4-Amino-4'-nitrostilbene	45	242–244 (245)[e]	3.866 (2H, br s), 6.693 (2H, d, J = 8.4 Hz), 6.94 (1H, d, J = 16.2 Hz), 7.18 (1H, d, J = 16.2 Hz), 7.376 (2H, d, J = 8.4 Hz), 7.567 (2H, d, J = 8.6 Hz), 8.186 (2H, d, J = 8.6 Hz).
4-(2-Phenylethyl)aniline	99	51–52 (52–54)[c]	2.75–2.93 (4H, m), 3.56 (2H, br s), 6.626 (2H, d, J = 8.3 Hz), 6.975 (2H, d, J = 8.3 Hz), 7.13–7.35 (5H, m)
4-Amino-2'-methylbiphenyl	95	oil (oil)[c]	2.286 (3H, s), 3.695 (2H, br s), 6.727 (2H, d, J = 8.4 Hz), 7.127 (2H, d, J = 8.4 Hz), 7.14–7.30 (4H, m).

[a] Calculation of coupling constants among 1,4-disubstituted phenyl hydrogens was simplified to determine only *ortho* interactions.
[b] Zheltov et al. (1970).
[c] Veschambre and Kergomard (1966).
[d] Weast and Grasselli (1989).
[e] Buckingham (1982).

TABLE 2

MUTAGENICITY OF AROMATIC AMINES IN *Salmonella typhimurium*

Compounds Doses (μmoles)	TA98 – S9 Revertants ± S.D.	Slope [a]	TA98 + S9 Revertants ± S.D.	Slope	TA100 – S9 Revertants ± S.D.	Slope	TA100 + S9 Revertants ± S.D.	Slope
4-Aminobiphenyl								
0	34 ± 4	0	65 ± 13	5652 ± 272	136 ± 10	0	156 ± 10	13653 ± 353
0.01	40 ± 10		138 ± 9		142 ± 9		277 ± 17	
0.033	50 ± 16		236 ± 22		152 ± 6		614 ± 9	
0.05	38 ± 5		339 ± 18		155 ± 5		911 ± 65	
0.066	48 ± 3		380 ± 76		150 ± 16		1068 ± 105	
0.1	43 ± 9		661 ± 81		147 ± 15		1505 ± 121	
0.33	53 ± 8		1188 ± 42		145 ± 6		2596 ± 132	
0.66	41 ± 7							
1	35 ± 8							
3	35 ± 10							
4,4'-Diaminobiphenyl								
0	29 ± 6	0	40 ± 8	62 ± 3	121 ± 22	0	151 ± 15	49 ± 6
0.1	32 ± 12		56 ± 9		132 ± 21		146 ± 23	
0.25	23 ± 2		87 ± 11		140 ± 11		183 ± 11	
0.5	29 ± 10		118 ± 10		137 ± 20		181 ± 30	
1	24 ± 6		149 ± 32		128 ± 15		217 ± 31	
2.5	28 ± 8		229 ± 18		153 ± 23		258 ± 10	
5	27 ± 4		357 ± 35		126 ± 11		242 ± 22	
10	27 ± 5		472 ± 14		116 ± 15		239 ± 25	
4-Amino-4'-chlorobiphenyl								
0	27 ± 8	0	45 ± 16	6092 ± 143	163 ± 10	0	168 ± 28	2891 ± 138
0.005			70 ± 9		159 ± 19		219 ± 41	
0.01			75 ± 9		158 ± 20		211 ± 31	
0.05			336 ± 18		159 ± 11		330 ± 45	
0.1	40 ± 8		645 ± 59		156 ± 10		493 ± 68	
0.3			1511 ± 142		151 ± 7		1050 ± 201	
0.33	45 ± 3							
0.66	43 ± 8							
1	50 ± 4							
3	41 ± 26							
4-Amino-4'-nitrobiphenyl								
0	32 ± 9	1304 ± 93	64 ± 9	10697 ± 427	124 ± 6	0	154 ± 17	1100 ± 112
0.001	38 ± 6		76 ± 13		120 ± 7		147 ± 14	
0.005	49 ± 9		106 ± 14		131 ± 20		173 ± 25	
0.01	[illegible]		[illegible]		[illegible]		[illegible]	
0.05	100 ± 11		543 ± 26		132 ± 14		214 ± 41	
0.1	129 ± 32		1136 ± 233		140 ± 31		266 ± 20	
0.33	226 ± 34							
0.66	233 ± 27							
1	258 ± 31							
3	311 ± 44							
4-Aminostilbene								
0	30 ± 5	37 ± 3	43 ± 7	3700 ± 179	121 ± 14	125 ± 25	128 ± 17	10059 ± 338
0.01	30 ± 8		58 ± 10		124 ± 10		156 ± 12	
0.033	30 ± 9		131 ± 30		115 ± 14		318 ± 32	
0.066	32 ± 8		298 ± 46		112 ± 17		741 ± 65	

TABLE 2 (continued)

Compounds Doses (μmoles)	TA98 – S9 Revertants ± S.D.	Slope [a]	TA98 + S9 Revertants ± S.D.	Slope	TA100 – S9 Revertants ± S.D.	Slope	TA100 + S9 Revertants ± S.D.	Slope
4-Aminostilbene								
0.1	41 ± 11		391 ± 77		135 ± 7		1094 ± 85	
0.3	42 ± 5		500 ± 118		155 ± 21		2277 ± 153	
0.33	51 ± 6							
0.5	41 ± 2							
0.66	49 ± 5							
1	70 ± 5							
3	64 ± 8							
4,4'-Diaminostilbene								
0	29 ± 6	0	45 ± 7	295 ± 27	121 ± 14	0	128 ± 17	123 ± 22
0.05	25 ± 4		64 ± 3		121 ± 6		152 ± 15	
0.1	34 ± 7		96 ± 15		121 ± 14		144 ± 24	
0.33	27 ± 11		146 ± 19		117 ± 10		173 ± 18	
0.66	35 ± 8		159 ± 35		131 ± 14		150 ± 26	
1	34 ± 7		142 ± 16		109 ± 20		167 ± 18	
4-Amino-4'-chlorostilbene								
0	31 ± 5	0	50 ± 10	14898 ± 426	115 ± 20	0	123 ± 17	6969 ± 215
0.005	30 ± 6		56 ± 19		136 ± 13		113 ± 11	
0.01	38 ± 6		73 ± 14		123 ± 24		162 ± 32	
0.033	41 ± 3		297 ± 24		132 ± 12		285 ± 31	
0.05	44 ± 10		628 ± 132		112 ± 15		396 ± 33	
0.066	59 ± 7		932 ± 98		133 ± 9		611 ± 71	
0.1	46 ± 11		1496 ± 117		125 ± 15		792 ± 70	
0.3	57 ± 11		2152 ± 68		107 ± 5		900 ± 25	
033	34 ± 2							
0.5	58 ± 13							
0.66	38 ± 3							
1	36 ± 5							
3	40 ± 5							
4-Amino-4'-nitrostilbene								
0	33 ± 6	1038 ± 94	53 ± 13	41958 ± 1127	107 ± 20	0	123 ± 24	6276 ± 249
0.001	48 ± 4		68 ± 8		88 ± 14		122 ± 21	
0.005	46 ± 12		160 ± 25		120 ± 9		158 ± 27	
0.01	68 ± 14		304 ± 23		119 ± 17		183 ± 23	
0.02	78 ± 13		748 ± 61		139 ± 17		257 ± 29	
0.03	75 ± 15		1320 ± 178		130 ± 38		334 ± 42	
0.033	114 ± 11		1685 ± 202		111 ± 9		326 ± 23	
0.05	90 ± 15		2012 ± 264		121 ± 14		432 ± 62	
0.066	106 ± 5		2627 ± 87		98 ± 14		363 ± 18	
0.1	113 ± 10		2105 ± 259		121 ± 13		477 ± 70	
0.33	155 ± 10							
0.66	166 ± 16							
1	199 ± 24							
4-(2-Phenylethyl)aniline								
0	30 ± 12	0	53 ± 18	82 ± 6	109 ± 10	0	116 ± 15	150
0.01	39 ± 5		74 ± 3		123 ± 5		144 ± 22	
0.1	31 ± 13		65 ± 9		120 ± 17		164 ± 47	
0.33	36 ± 6		85 ± 11					
0.5	28 ± 5		91 ± 9		109 ± 14		211 ± 73	
0.66	48 ± 2		112 ± 4					
1.0	36 ± 14		140 ± 18		107 ± 17		284 ± 106	
1.5					75 ± 8		348 ± 34	
2.0	3 ± 4		131 ± 25		41 ± 18		325 ± 17	

TABLE 2 (continued)

Compounds Doses (μmoles)	TA98 – S9 Revertants ± S.D.	Slope [a]	TA98 + S9 Revertants ± S.D.	Slope	TA100 – S9 Revertants ± S.D.	Slope	TA100 + S9 Revertants ± S.D.	Slope
4,4'-Ethylenebis(aniline) [b]								
0	32 ± 10	0	32 ± 9	17 ± 2	98 ± 21	0	82 ± 15	60 ± 6
0.01	33 ± 3		35 ± 4		102 ± 12		95 ± 10	
0.1	27 ± 5		38 ± 7		112 ± 14		100 ± 6	
1	35 ± 8		52 ± 3		115 ± 9		174 ± 25	
2	31 ± 5		68 ± 6		114 ± 16		203 ± 6	
5	30 ± 5		69 ± 6		134 ± 9		244 ± 21	
2-Aminofluorene								
0	28 ± 3	400 ± 19	62 ± 12	31518 ± 1313	125 ± 11	0	149 ± 15	17697 ± 588
0.0005			87 ± 21		134 ± 25		170 ± 17	
0.001			89 ± 22		120 ± 18		170 ± 10	
0.005			185 ± 20		149 ± 16		231 ± 34	
0.01			336 ± 33		130 ± 14		273 ± 48	
0.02			547 ± 53		137 ± 20		399 ± 14	
0.04			1300 ± 202		137 ± 21		876 ± 91	
0.05			1665 ± 521		130 ± 14		1032 ± 193	
0.1	84 ± 19							
0.33	167 ± 33							
0.66	323 ± 34							
1	425 ± 69							
3	371 ± 51							
2,7'-Diaminofluorene								
0	28 ± 3	563 ± 44	67 ± 15	7588 ± 271	125 ± 10	28 ± 6	145 ± 14	659 ± 12
0.0005			75 ± 15		130 ± 19		163 ± 12	
0.001			70 ± 16		120 ± 18		152 ± 11	
0.005			105 ± 29		117 ± 20		163 ± 15	
0.01			138 ± 8		122 ± 6		150 ± 21	
0.025			291 ± 20					
0.05			442 ± 75		128 ± 18		190 ± 31	
0.1	91 ± 8		533 ± 103		151 ± 14		267 ± 30	
0.33	241 ± 21				141 ± 17		418 ± 49	
0.66	445 ± 127				149 ± 15		593 ± 32	
1	580 ± 160				149 ± 10		804 ± 30	
3	1102 ± 243							
4-Amino-2'-methylbiphenyl								
0	36 ± 9	0	53 ± 9	760 ± 56	100 ± 11	0	149 ± 13	1436 ± 86
0.05	33 ± 8		115 ± 13		94 ± 10		236 ± 45	
0.1	37 ± 10		202 ± 37		100 ± 9		409 ± 54	
0.3	41 ± 20		[illegible]		[illegible]		[illegible]	
0.5	43 ± 16		445 ± 87		113 ± 17		875 ± 117	
1.0	39 ± 20		556 ± 93		101 ± 21		1163 ± 65	

[a] Values are the calculated slopes (rev/μmole) of the linear portion of the dose–response curve determined by at least two independent experiments.

[b] Data from Messerly et al. (1987).

Notes to table 3:

[a] Total chromatid and chromosome gaps at each concentration per 100 cells were recorded but not included as aberrations.

[b] Cells with at least 1 aberration. Results are for 4 animals at each concentration (100 cells/animal) except where noted.

[c] Results at each concentration were compared to those of the control using Dunnett's test (* $P < 0.05$ and ** $P < 0.01$).

[d] 1 of the 4 animals died at this concentration.

[e] All 4 animals died at this concentration.

TABLE 3

CHROMOSOMAL ABERRATIONS INDUCED BY AROMATIC AMINES IN BONE MARROW CELLS OF MICE

Treatment (mg/kg)	Gaps [a]	Aberrations/cell		Aberrant cells (%) [b] (mean ± S.D.) [c]	Mitotic indices (%) (mean ± S.D.) [c]
		Chromatid type	Chromosome type		
Solvent control					
DMSO	3	0.010	0.000	1.00 ± 0.82	3.51 ± 0.3
4-Aminobiphenyl					
25	3.25	0.028	0.000	2.75 ± 0.96 *	3.62 ± 0.37
100 [d]	6.67	0.067	0.003	7.00 ± 1.00 **	1.69 ± 0.25 **
4,4'-Diaminobiphenyl					
10	5.50	0.013	0.000	1.25 ± 0.50	3.71 ± 0.16
25	6.25	0.020	0.003	2.25 ± 0.50	2.69 ± 0.19 **
50	6.75	0.035	0.003	3.75 ± 0.50 **	2.35 ± 0.20 **
100	6.25	0.043	0.008	5.00 ± 0.82 **	2.63 ± 0.33 **
125	7.75	0.038	0.023	6.00 ± 0.82 **	2.62 ± 0.41 **
4-Amino-4'-chlorobiphenyl					
25	3.00	0.033	0.000	3.00 ± 0.82 **	2.31 ± 0.16 **
100 [e]					
4-Amino-4'-nitrobiphenyl					
25	3.00	0.018	0.000	1.75 ± 0.50	2.57 ± 0.40 **
100	4.00	0.033	0.008	4.00 ± 0.82 **	1.77 ± 0.24 **
4-Aminostilbene					
10	3.00	0.030	0.000	2.75 ± 0.50 **	2.77 ± 0.38 *
25	5.25	0.085	0.013	8.50 ± 1.29 **	2.65 ± 0.35 **
100 [d]	11.00	0.303	0.023	20.33 ± 2.08 **	1.50 ± 0.24 **
4,4'-Diaminostilbene					
25	5.25	0.023	0.000	2.25 ± 0.50	2.85 ± 0.38 *
100	4.50	0.025	0.000	2.50 ± 1.00 *	2.58 ± 0.36 **
4-Amino-4'-chlorostilbene					
25	2.50	0.020	0.000	2.00 ± 0.82	2.66 ± 0.09 **
100	6.25	0.028	0.005	3.25 ± 0.50 **	2.28 ± 0.08 **
4-Amino-4'-nitrostilbene					
25	4.50	0.028	0.000	2.75 ± 0.50 **	2.31 ± 0.31 **
100	3.75	0.043	0.008	5.00 ± 0.82 **	1.95 ± 0.29 **
4-(2-Phenylethyl)aniline					
100	4.25	0.028	0.000	2.75 ± 0.96 *	2.31 ± 0.39 **
100					
4,4'-Ethylenebis(aniline)					
100	3.00	0.020	0.000	2.00 ± 0.82	2.67 ± 0.29 **
2-Aminofluorene					
100	6.00	0.048	0.005	5.25 ± 1.26 **	2.70 ± 0.47 *
2,7-Diaminofluorene					
100	3.75	0.045	0.003	4.75 ± 0.96 **	2.57 ± 0.36 **
4-Amino-2'-methylbiphenyl					
100	2.50	0.033	0.008	4.00 ± 1.16 **	2.66 ± 0.36 **
Positive control DMBA (100 mg /kg)	11.25	0.115	0.023	13.25 ± 0.96 **	2.13 ± 0.12

scored with the aid of an Artex counter (Dynatech Labs., Chantilly, VA) which had been calibrated against manually scored plates.

Chromosomal aberrations (CA)

Test chemicals in DMSO at the concentrations shown in Table 3 were administered i.p. as single doses to each of 4 mice. Negative control mice were injected with 2 ml/kg DMSO while DMBA was used as a positive control at a dose of 100 mg/kg in DMSO. CA assays were conducted as previously described (Giri et al., 1989) with a fixation time of 24 h after injection which is consistent with the protocol of Preston et al. (1987). This fixation time was found to be an optimal time for benzidine after testing at 6, 12 and 24 h. All the slides were coded and observed by a single observer. 100 well-spread metaphase cells were scored per animal from each of 4 animals at each concentration tested. Mitotic indices were calculated from 1000 cells/animal and expressed as percentages. CA were scored following the method of WHO (1985) and Preston et al. (1987). The aberration frequencies per cell for chromatid and chromosome types were calculated. Gaps were recorded (Table 3) but not included in the frequency of aberrations per cell.

Results and discussion

The in vitro results with Salmonella strains TA98 and TA100 are summarized in Table 2. As noted in our previous study of benzidine analogs and from the well documented need for metabolic activation of aromatic amines to produce alkylating agents (Beland and Kadlubar, 1985), there was only limited activity in either strain without S9 activation. The major exceptions were the two nitro derivatives, where the presence of a nitro reductase system in TA98 (Nohara et al., 1985) could explain the moderate mutagenicities of these compounds in this strain without S9 activation.

In general, π-orbital conjugation between the two phenyl rings is an important factor for mutagenicity in both strains with S9 activation. There is an increase in mutagenicity with an increase in planarity and its resulting increase in conjugation for the series 4-amino-2'-methylbiphenyl, 4-aminobiphenyl and 2-aminofluorene. This is a series in which the parent compounds for these amines, 2-methylbiphenyl, biphenyl and fluorene have an angle between the two rings of 58, 23 and 0° respectively (Suzuki, 1959a, b, c). Likewise, 2,7-diaminofluorene is more active than benzidine. Also, the extended conjugation of the stilbenes over their corresponding biphenylamines for the most part led to an increase in mutagenicity. The exception, in this regard, was the greater mutagenicity of 4-aminobiphenyl over 4-aminostilbene in both strains with activation. Elimination of conjugation between the phenyl rings in the stilbenes by saturation of the carbon–carbon double bond clearly resulted in a decrease in mutagenicity for 4-(2-phenylethyl)aniline and 4,4'-ethylenebis(aniline) compared to the parent stilbenes.

The results in Table 2 indicate that the substituents in the 4'-position of both the biphenyl series and the stilbene series have an electronic effect on mutagenicity. With increasing electron withdrawing capacity of these substituents, there is increasing mutagenicity in the order of $NH_2 < H < Cl < NO_2$. This relationship only holds in the tests with TA98 which detects frameshift mutations.

The in vivo genotoxicity of the aromatic amines under consideration as measured by CA in the bone-marrow cells of mice is summarized in Table 3. A dose–response relationship was established for the acute genotoxicity of benzidine in the range from 0 to 125 mg/kg of body weight. The acute genotoxicity of the other amines were compared to that of benzidine at 25 and/or 100 mg/kg. With the exception of 4,4'-ethylenebis(aniline), all compounds showed a significant ($p < 0.05$) increase in CA, at least at the 100 mg/kg dose compared to the negative control.

Relative mutagenicities in the Ames test did not correlate to in vivo CA results. The effect of π-orbital conjugation was less pronounced in vivo than in vitro. Also, the increased effect on genotoxicity with increases in the electron withdrawing ability of substituents was not evident in vivo as it was in vitro. For example, the high mutagenicity for 4-amino-4'-nitrobiphenyl in comparison to the other biphenyls is in contrast to its lower ranking for CA results. The outstanding difference in comparing the in vitro and in vivo

results was the exceptionally high CA values for 4-aminostilbene. The CA results of 4-aminostilbene was much greater than those for any of the other compounds tested. This includes the well established carcinogen, DMBA, our positive control. All the conjugated mono-amino compounds had relatively high CA values.

The significantly higher CA values for the mono-amino compounds over their corresponding di-amino derivatives in the stilbene ($p < 0.001$) and biphenyl ($p < 0.05$) series led to a review of the literature in regards to such comparisons. While there is an extensive literature on the genotoxicity of 4-aminobiphenyl, benzidine and 2-aminofluorene, this is not true for 2,7-diaminofluorene nor 4-amino- or 4,4'-diaminostilbene (Sweet, 1987). Direct comparisons of these pairs of mono- and di-substituted amines are for the most part limited to the two biphenyl compounds and usually represent in vitro tests. As was confirmed in the present study, it is well established that 4-aminobiphenyl is more mutagenic than benzidine in the Ames test with standard rat S9 activation (McCann et al., 1975; Bos et al., 1982; Nohara et al., 1985; Messerly et al., 1987). However, when activation in the Ames test was with rat liver hepatocytes, benzidine exhibited greater mutagenicity than 4-aminobiphenyl (Bos et al., 1982).

As is true in the present study, it has also been previously reported that 2-aminofluorene in the Ames test with rat S9 activation has greater mutagenicity than 2,7-diaminofluorene (McCann et al., 1975; Vance et al., 1987). The same relationship holds when the activation is with microsomes from Drosophila (Nix et al., 1981) or with microsomes from Salamanders (Anderson et al., 1982). Other in vitro studies of benzidine and 4-aminobiphenyl have indicated 4-aminobiphenyl to be more genotoxic than benzidine on the basis of a DNA repair test (Althaus and Pitot, 1983) and by an alkaline elution assay with rat hepatocytes (Sina et al., 1983). However, these two compounds had about an equal response in a DNA damage-alkaline elution assay with Chinese hamster cells (Swenberg et al., 1976). In a DNA-excision repair assay with rat hepatocytes, benzidine gave a greater rise in unscheduled DNA synthesis than 4-aminobiphenyl (Brouns et al., 1979).

Prior reports of the in vivo genotoxicity of the mono-amino vs. di-amino compounds examined in the present study also appear to be restricted to comparisons of benzidine and 4-aminobiphenyl. In their review of the literature, Arcos and Argus (1974) reached the conclusion that 4-aminobiphenyl is more genotoxic in vivo than benzidine as is the case for our bone-marrow studies. However, Parodi et al. (1982) calculated a greater oncogenic potency index for benzidine than for 4-aminobiphenyl and Bos et al. (1982) reported greater genotoxicity for benzidine compared to 4-aminobiphenyl in a host-mediated assay. A somewhat greater response for induced sister-chromatid exchange in vivo in the bone marrow of chinese hamsters has been reported for benzidine dihydrochloride compared to 4-aminobiphenyl at 50 mg/kg (Neal and Probat, 1983). At this dose, they also reported a similar effect to that of 4-aminobiphenyl for 2-aminofluorene.

In general, literature reports of bone-marrow tests for aromatic amines in vivo are often inadequate (Mirkova and Ashby, 1988) although they are desirable to supplement Ames test results (Shelby, 1988). The present study extends such comparisons of results from the Ames test to those in vivo in bone marrow. The most evident difference in vivo was the exceptionally high genotoxicity of 4-aminostilbene. This, together with the relatively high in vivo response for 4-aminobiphenyl and 2-aminofluorene, points out the potential for increased genotoxicity for those aromatic amines related to benzidine but without substitution in the *para'* position.

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References

Althaus, F.R., and H.C. Pitot (1983) A rapid technique for the quantitation of DNA-repair synthesis in the hepatocyte/DNA-repair test for chemical carcinogens. Ann. N.Y. Acad. Sci., 407, 463–466.

Anderson, R.S., J.A. Döös and F.L. Rose (1982) Differential ability of *Ambystoma tigrinum* hepatic microsomes to produce mutagenic metabolites from polycyclic aromatic hydrocarbons and aromatic amines. Cancer Lett., 16, 33–41.

Arcos, J.C., and M.F. Argus (1974) Chemical Induction of Cancer, Vol. IIB, Academic Press, New York, pp. 33–51.

Beaven, G.H., P.B.D. De La Marc, M. Hassan, E.A. Johnson and N.V. Klassen (1961) The kinetics and mechanisms of aromatic halogen substitution, Part X. Products in the chlorination of biphenyl in acetic acid, J. Chem. Soc., 2749–2753.

Beland, F.A., and F.F. Kadlubar (1985) Formation and persistence of arylamine DNA adducts in vivo, Environ. Health Perspect., 62, 19–30.

Bos, R.P., R. van Doorn, E. Yih-van de Hurk, P.J.L. van Gemert and P.Th. Henderson (1982) Comparison of the mutagenicities of 4-aminobiphenyl and benzidine in the Salmonella/microsome, Salmonella/hepatocyte and host-mediated assays, Mutation Res., 93, 317–325.

Brouns, R.E., M. Poot, R. de Vrind, Th.v. Hoek-Kon and P.Th Henderson (1979) Measurement of DNA-excision repair in suspension of freshly isolated rat hepatocytes after exposure to some carcinogenic compounds, Mutation Res., 64, 425–432.

Buckingham, J. (1982) Dictionary of Organic Compounds, 5th edn., Chapman and Hall, New York.

France, H., I.M. Heilbron and D.H. Hey (1939) Studies in the terphenyl series. Part II. Hydroxy- and methyl-*p*-terphenyls, J. Chem. Soc., 1283–1287.

Geerts, J.P., and R.H. Martin (1960) Synthese dans le domanine des compose polycycliques aromatiques, Bull. Soc. Chim. Belges, 69, 563–569.

Giri, A.K., E.A. Messerly and J.E. Sinsheimer (1989) Sister-chromatid exchange and chromosome aberrations for 4 aliphatic epoxides in mice, Mutation Res., 224, 253–261.

Haley, T.J. (1975) Benzidine revisited: A review of the literature and problems associated with the use of benzidine and its congeners, Clin. Toxicol., 8(1), 13–42.

Maron, D.M., and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test; Mutation Res., 113, 173–215.

McCann, J., E. Choi, E. Yamasaki and B.M. Ames (1975) Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals, Proc. Natl. Acad. Sci. (U.S.A.), 72, 5135–5139.

Messerly, E.A., J.E. Fekete, D.R. Wade and J.E. Sinsheimer (1987) Structure–mutagenicity relationships of benzidine analogues, Environ. Mol. Mutagen., 10, 263–274.

Mirkova, E., and J.Ashby (1988) Activity of the human carcinogen benzidine and 2-naphthylamine in male mouse bone marrow micronucleus assays, Mutagenesis, 3, 437–439.

Natarajan, A.T., and G. Obe (1986) How do in vivo mammalian assays compare to in vitro assays in their ability to detect mutagens?, Mutation Res., 167, 189–201.

Neal S.B., and G.S. Probat (1983) Chemically-induced sister-chromatid exchange in vivo in bone marrow of Chinese hamsters, Mutation Res., 113, 33–43.

Nix, C.E., B. Brewen and J.L. Epler (1981) Microsomal activation of selected polycyclic aromatic hydrocarbons and aromatic amines in *Drosophila melanogaster*, Mutation Res., 88, 291–299.

Nohara, M., T. Hirayama, Y. Fujioka, S. Ozasa, E. Ibuki and S. Fukui (1985) Relationship between mutagenic potency in *Salmonella typhimurium* and chemical structure of amino and nitro-substituted biphenyls, Mutation Res., 149, 9–15.

Parodi, S., M. Taningher and L. Santi (1982) Alkaline elution in vivo: fluorometric analysis in rats quantitative predictivity of carcinogenicity, as compared with other short-term tests, in: B.A. Bridges, H.E. Butterworth and I.B. Weinstein (Eds.), Banbury Report 13, Indicators of Genotoxic Exposure, Cold Spring Harbor Laboratory, New York, pp. 137–155.

Porter, H.K. (1973) The Zinin reduction of nitroarenes, Organic Reactions, 20, 455–481.

Preston, R.J., B.J. Dean, S. Galloway, H. Holden, A.F. McFee and M. Shelby (1987) Mammalian in vivo cytogenetic assays: Analysis of chromosome aberrations in bone marrow cells, Mutation Res., 189, 157–165.

Shelby, M.D. (1988) The genetic toxicity of human carcinogens and its implications, Mutation Res., 204, 3–15.

Sina, J.F., C.L. Bean, G.R. Dysart, V.I. Taylor and M.O. Bradley (1983) Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential, Mutation Res., 113, 357–391.

Suzuki, H. (1959a) Relations between electronic absorption spectra and spatial configurations of conjugated systems. I. Biphenyl, Bull. Chem. Soc. (Japan), 32, 1340–1350.

Suzuki, H. (1959b) Relations between electronic absorption spectra and spatial configurations of conjugated systems, II. *o*-Alkyl and *o,o'*-dialkyl biphenyls, Bull. Chem. Soc. (Japan), 32, 1350–1356.

Suzuki, H. (1959c) Relations between electronic absorption spectra and spatial configurations of conjugated systems, III. *o,o'*-Bridged biphenyls, Bull. Chem. Soc. (Japan), 32, 1357–1361.

Sweet, D.V. (1987) Registry of Toxic Effects of Chemical Substances, 1985–86 edn., Vol. 2, U.S. Government Printing Office, Washington, DC.

Swenberg, J.A., G.L. Petzold and P.R. Harboch (1976) In vitro DNA damage/alkaline elution assay for predicting carcinogenic potential, Biochem. Biophys. Res. Commun., 72, 732–738.

U.S. Federal Register (1974) (part III) No. 20, Carcinogens Occupational Health Safety Standards, 39, 3755–3797.

Vance, W.A., Y.Y. Wang and H.S. Okamoto (1987) Disubstituted amino-, nitroso-, and nitrofluorenes: a physicochemical basis for structure–activity relationships in *Salmonella typhimurium*, Environ. Mutagen., 9, 123–141.

Veschambre, H., and A. Kergomard (1966) Transmission of electronic effects in substituted trans-stilbenes, Bull. Soc. Chem. (France), 1, 336–342.

Weast, R.C., and J.G. Grasselli (1989) Handbook of Data on Organic Compounds, 2nd edn., CRC Press, Boca Raton.

World Health Organization (1985) Environmental Health Criteria, 46. Guidelines for the Study of Genetic Effects in Human Populations, WHO, Geneva.

Zavon, M.R., V. Hoegg and E. Bingman (1973) Benzidine exposure as a cause of bladder tumors, Arch. Environ. Health, 27, 1–8.

Zheltov, A.Y., V.Y. Rodionov and B.I. Stepanov (1970) Investigations on aromatic disulfides, III. Basicity and structure of substituted 4- and 5-aminobiphenylene-2,2'-disulfide, Zhur. Org. Khim., 6, 2562–2569.