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Mutagenic Activities of Aromatic Amines in *Salmonella typhimurium* and its Effect on Rat Drug Metabolizing Enzymes

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ABSTRACT. The aromatic amines including o-toluidine, 4-chloro-o-toluidine, 4-4'-methylene dianiline (MDA), 4,4'-methylene bis(2-chloroaniline) (MOCA) and its three possible substitutes, ethacure 300, cyanacure, and polacure 740 M, were used to determine the mutagenic activities in *Salmonella typhimurium* strains TA98 and TA100. The mutagenic activities of these chemicals were compared with their effects on ethoxyresorufin o-deethylase (EROD) activity and aldrin epoxidase (AE) activity in rat liver. The chemicals o-toluidine, 4-chloro-o-toluidine, MDA, MOCA and its substitutes, ethacure 300 and cyanacure, showed mutagenic activities, while polacure 740 M showed no mutagenic activity . All the mutagens caused an increase in EROD activity , while polacure 740 M showed no appreciable increase in EROD activity . Thus, there was excellent correlation between mutagenicity and EROD induction. This supports the conclusion that the ability of a chemical to induce EROD bears some relationship to its carcinogenic potential.

4,4'-Methylene-bis-(2-chloroaniline) (MOCA) is a curing agent used in the manufacutre of polyurethane. Workers are potentially exposed (Ward *et al.* 1987). There are reliable data indicating that MOCA behaves as an experimental carcinogen. It has been reported to be responsible for the occurrence of liver and lung tumors in mice and rats (Russfield *et al.* 1975, Kommineni *et al.* 1979) and the development of bladder tumors in dogs (Stula *et al.* 1977). Consequently, MOCA is considered a human carcinogen (Ward 1982, IARC 1987) because of the similarity of pathways of metabolism to those in experimental animals (Morton *et al.* 1988, Butler *et al.* 1989) and a reported excess incidence of bladder cancer in exposed workers (Ward *et al.* 1990). MOCA showed a

mutagenic activity in the presence of rat liver Sg mix in a salmonella/mammalian microsome assay (Rao *et al.* 1982, Hesbert *et al.* 1985). The interaction of some intermediates with DNA bases appears to be the initial step of mutagenesis.

There are three possible replacements currently available for the industrial application(s) of MOCA. These are ethacure 300, cyanacure,and polacure 740 M. As little is known about the genotoxicity of these compounds, they require investigation for their effects on rat liver drug-metabolizing enzymes. Furthermore, they provide a convenient group of unknowns to explore further the correlation between carcinogenic potential and effects on the drug-metabolizing enzymes. Thus, studies to determine the mutagenicity of these chemicals (Fig. 1) and their effects on ethoxyresorufin o-deethylase (EROD) and aldrin expoxidase (AE) activities in rat liver were undertaken.

Materials and Methods

Chemicals

MOCA and MDA were obtained from Tokyo Kasei, Japan, Ethacure 300, cyanacure and polacure 740 M were obtained from Research Plus, Inc., Bayonne, NJ. o-Toluidine, 4-chloro-o-toluidine and 2-nitrofluorene were purchased from Aldrich Chemical Company, Milwaukee. WI. Polychlorinated biphenyl (Aroclor 1254) was obtained from Analabs, Inc., New Haven, CT. Enzymes and cofactors Bayonne, were purchased from Sigma Chemical Co. and Boehringer Mannheim.

Animals

Male Sprague-Dawley rats weighing 120-150 g. were injected with MOCA, ethacure 300, cyanacure, polacure 740 M, MDA, o-toluidine, and 4-chloro-o-toluidine, dissolved in dimethyl sulphoxide (DMSO). To determine the effects of these compounds on EROD and AE activities, they injected i.p. at doses 20 and 200 mg/kg, so that the rats received 1 ml/kg of the DMSO, which was the same for control rats. Administration was once daily for three consecutive days and the rats were killed 24 h after the last dose.

Preparation of Hepatic S_9 and Microsomal Fraction

The rats were injected with Aroclor 1254 (500 mg/kg i.p.). The liver homogenate fraction (S_9) was prepared according to the method of Maron and Ames (1983). To prepare the microsomal fraction, the 9000 g supernatant fraction (S_9) was further centrifuged at 105,000 g for 60 min. and the pellet was resuspended in the half volume of cold 0.15 M KCI. Both fractions (S_9 and microsomes) were frozen in dry ice plus acetone and stored at –80°C (Maron and Ames 1983).

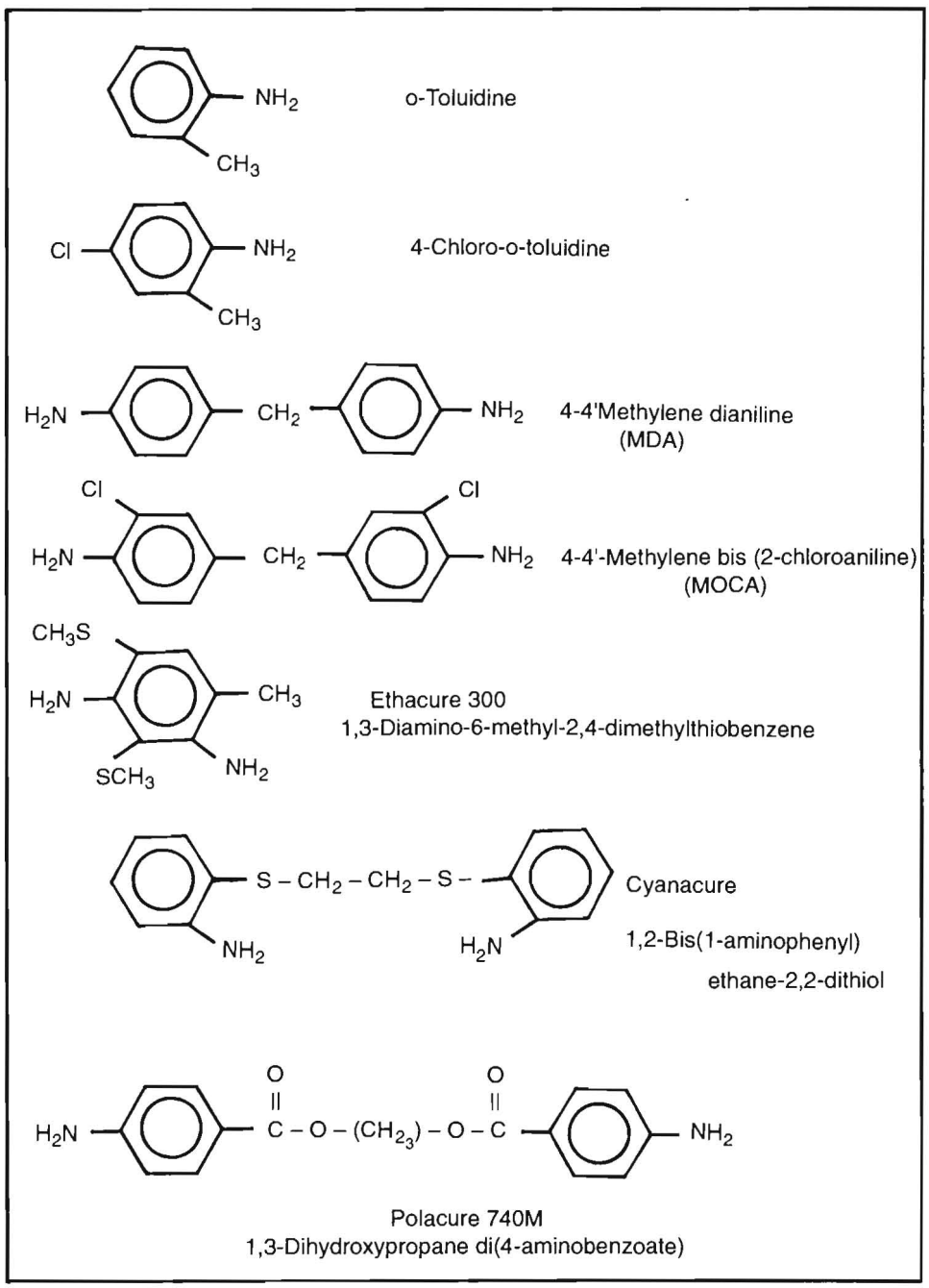

Fig. 1. The chemical structure of the seven related aromatic amines.

Mutagenesis Assay

Salmonella typhimurium TA98 and TA100 were kindly provided by Dr. B. N. Ames (Berkeley, CA). Mutagenicity assays were performed essentially as described by Maron and Ames (1983). Bacteria (100 µl, density of 1-2 x 10^9 cells/ml), test compound (add in 100 µl of DMSO) and S9 mix (0.5 ml of respective concentrations) were added to 2 ml of top agar supplementer with a trace of histidine and biotin. 2-Nitrofluorene was used to validate the mutagenic responses of both strains in these assays. Triplicate plates at each dose level were incubated at 37°C for 72 h before counting revertant colonies with Artek model 880 counter.

Microsomal Enzyme Assays

The protein contents were determined by the method of Lowry *et al.* (1951). Ethoxyresorufin o-deethylation was determined by the procedure of Prough *et al.* (1978). Aldrin epoxidation was determined according to the procedure of Wolff *et al.* (1979).

Statistics

"t" Test was applied in order to compare the enzyme activity between the control and the treated animals. Significant level was set at $P < 0.05$.

Results

The mutagenic activity of structurally related aromatic amines such as o-toluidine, 4-chloro-o-toluidine, MDA, MOCA, ethacure 300, cyanacure and polacure 740 M were assayed in *S. typhimurium* strains TA98 and TA100, in the presence of a liver homogenate activation system, liver S_9 fraction. The maximum concentrations were determined by a preliminary toxicity test with TA100 on minimal agar plates. The data are presented in Table (1). o-Toluidine and 4-chloro-o-toluidine did not increase the revertant number of strain TA100. However, they showed mutagenic activities in TA98. MOCA was mutagenic in the presence of metabolic activation in both TA98 and TA100. It produced a doubling in TA100 revertants at 25 µg/plate, whereas MDA produced a doubling in the same strain at 50 µg/plate. Ethacure 300 induced an 8-fold dose-related increase in TA98 revertants in the presence of S_9 mix. The response in TA100 was much weaker, but still resulted in approximate dose-related revertant colonies.

The mutagenicity of cyanacure, like that of ethacure 300, was S_9 dependent, but contrasted with respect to strain specificity. The activity of this compound could be demonstrated only in TA100, where a dose-related 4-fold increase in revertants was seen over the concentration range of 50-200µg/plate. Polacure 740 M was not mutagenic in either TA98 or TA100, even at the highest concentration of 400 µg/plate.

Table 1. Mutagenic activity of structurally related aromatic amines in *S. typhimurium* TA 98 and TA100

Chemical	Dose (μg/plate)	Revertant Colonies/Plate	
		TA98	TA100
o-Toluidine	0.25	73 ± 4	128 ± 8
	0.50	96 ± 6	131 ± 9
	0.75	113 ± 9	123 ± 9
	1.00	124 ± 10	121 ± 10
4-Chloro-o-toluidine	0.25	72 ± 4	119 ± 8
	0.50	106 ± 9	125 ± 9
	0.75	15 ± 13	131 ± 10
	1.00	132 ± 11	127 ± 11
MDA	25	68 ± 4	152 ± 11
	50	122 ± 9	234 ± 15
	75	179 ± 14	378 ± 18
	100	164 ± 12	447 ± 22
MOCA	25	131 ± 9	235 ± 29
	50	212 ± 14	402 ± 34
	75	334 ± 29	608 ± 43
	100	387 ± 25	761 ± 38
Ethacure 300	50	71 ± 5	163 ± 11
	100	122 ± 9	240 ± 19
	200	175 ± 12	272 ± 24
	400	201 ± 10	205 ± 13
Cyanacure	50	43 ± 3	173 ± 14
	100	48 ± 2	247 ± 16
	200	42 ± 2	329 ± 28
	400	47 ± 4	280 ± 18
Polacure 740M	50	43 ± 2	130 ± 10
	100	51 ± 3	124 ± 9
	200	47 ± 2	123 ± 10
	400	45 ± 2	121 ± 9
2-Nitrofluorene	0.25	619 ± 29	431 ± 21
	0.50	1123 ± 59	721 ± 43
	0.75	1604 ± 93	811 ± 63
	1.00	1781 ± 86	983 ± 59

Effects on Microsomal Activities

The effects of seven structurally related aromatic amines on EROD andAE activities are shown in Table (2). o-Toluidine, 4-chloro-o-toluidine and MDAshowed a significant increase in EROD activity. MOCA, ethacure 300, and cyanacure caused a 36–, 18–, and 6-fold induction in EROD activity, respectively. Polacure 740 M caused no induction of EROD activity. MOCA caused an increase in AE activity, whereas ethacure 300, cyanacure, and polacure 740 M did not cause a significant effect on AE activity.

Table 2. Effects of structurally related aromatic amines on ethoxyresorufin-o-deethylase (EROD) and aldrin epoxidase (AE) activity in rat liver microsomes

Chemical	Dose (mg/kg)	Activity (n mol/min/mg pr otein)	
		FROD 98	AE 100
Control		0.021 ± 0.003*	2.97 ± 0.27
o-Toluidine	20 200	0.038 ± 0.007 0.129 ± 0.031*	3.42 ± 0.72 5.62 ± 1.09*
4-Chloro-o-toluidine	20 200	0.029 ± 0.003 0.142 ± 0.012*	3.21 ± 0.91 3.34 ± 0.56
MDA	20 200	0.182 ± 0.057* 0.567 ± 0.204*	1.42 ± 0.37* 0.85 ± 0.09*
MOCA	20 200	0.213 ± 0.082* 0.746 ± 0.343*	0.72 ± 0.03* 0.31 ± 0.02*
Ethacure 300	20 200	0.048 ± 0.007 0.378 ± 0.037*	3.41 ± 0.63 2.75 ± 0.25
Cyanacure	20 200	0.043 ± 0.009* 0.131 ± 0.022	2.63 ± 0.37 3.24 ± 0.82
Polacure 740M	20 200	0.034 ± 0.00 0.031 ± 0.00	3.32 ± 0.67 2.74 ± 0.43

a) Values are mean ± SD.

*) Significantly different to control.

Discussion

The polyurethane curative, MOCA, which is currently widely used, was found to be mutagenic (Roa *et al.* 1982, Hesbert *et al.* 1985) and carcinogenic (Russfield *et al.* 1975, Kommineni *et al.* 1979, IARC 1987). Of the chemicals that are currently available as substitute curatives in polyurethane manufacture, ethacure 300, cyanacure and polacure 740 M were used. Ethacure 300 was mutagenic in both bacterial strains TA98 and TA100, whereas cyanacure was positive only in TA 100. Polacure 740M was found to be negative in both strains.

The induction of EROD activity by MOCA, ethacure 300, and cyanacure, but not polacure 740M, is consistent with their mutagenic effects. Furthermore, it is of interest that MOCA was clearly the strongest inducer of EROD activity and the strongest mutagen, while polacure 740M was clearly negative for both.

Furthermore, our studies support an earlier report (FAO/WHO 1985) for the mutagenic activities of o-toluidine and 4-chloro-o-toluidine, and also for effects of these two chemicals on EROD and AE activities (Leslie *et al.* 1988). The carcinogenicity of o-toluidine and 4-chloro-o-toluidine have been documented in the literature (IARC 1978, FAO 1980, Rubbino *et al.* 1982). The positive results with the genotoxicity assay of MDA is in line with published data (IARC 1980, Rao *et al.* 1982, Mori *et al.* 1988, McQueen and Williams 1990). Our studies also determined that MDA increased EROD activity, while the activity of AE was decreased.

The carcinogenicity of MDA are referenced in the literature (Weisburger *et al.* 1984, McQueen and Williams 1990). For ease of comparison, the chemicals and their structures are given in Fig. (1), and their mutagenic and carcinogenic properties along with effects on EROD and AE activities are provided in Table (3). From the information in Table (3), it can be appreciated that there is no consistent relationship between mutagenicity or carcinogenicity and a decrease in AE activity. On the other hand, for this group of aromatic amines, there is an excellent qualitative correlation between mutagenic or carcinogenic properties and the ability to induce EROD activity in rat liver. Since AE is a sensitive indicator for cytochrome P-450 activity (Wolff *et al.* 1979) and EROD a sensitive marker enzyme for cytochrome P-448 activity (Delaforge *et al.* 1980), this supports the conclusion that cytochrome P-448 (Cytochrome P-450) converts the chemical carcinogenesis to reactive electrophiles, which react covalently with cellular macromolecules giving rise to mutagenicity and carcinogenicity (Nebert *et al.* 1985, Ioannides and Parke 1987).

Table 3. Comparison of seven structurally related aromatic amines for mutagenicity , carcinogenicity, and effects on EROD and AE activities

Chemical	Mutagenicity	Carcinogenicity*	EROD	AE
o-Toluidine	+	+	up	up
4-Chloro-o-toluidine	+	+	up	no change
MDA	+	+	up	down
MOCA	+	+	up	down
Ethacure 300	+	?	up	no change
Cyanacure	+	?	up	no change
Polocure 740 M	–	?	no change	no change

*) Carcinogenicity data taken from the literature. See text for appropriate references.

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النشاطات السرطانية للمركبات الحلقية الأمينية في *Salmonella typhimurium*

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تم استخدام المركبات الأمينية : ١- تلودين و ٤- كلورو- ١- تلودين و٤ ، ٤ - مثلين ثنائي أنلين ، و ٤ ، ٤ -مثلين بس (٢ - كيلورو أنلين) وثلاثة مشتقات آخرى وهي :

أثكيور ٣٠٠ وسيناكيور وبولاكيور ٧٤٠ م لدراسة النشاطات السرطانية في *Salmonella typhimurium* .

وقد تم مقارنة النشاطات السرطانية لهذه المركبات مع تأثيرها على أنزيم إثوكسي سورفن ١- دثليز وكذلك انزيم الدرن أبوكسيدز في كبد الفئران .

وقد بينت الدراسة أن جميع هذه المركبات بخلاف مركب بولاكيور ٧٤٠م قد أظهرت نشاطات سرطانية حيث أدت إلى ارتفاع في نسبة نشاط أنزيم إثوكسي سورفن ١-دثليز .هذا ويبين البحث العلاقة بين النشاط السرطاني والحث الحيوي لأنزيم إثوكسي سورفن ١-دثليز .