Effects of Microsomal Enzyme Induction on Toxicity of *p*-*N*,*N*-Bis(2-chloroethyl)aminophenyl Alkyl Ethers in Mice and Survival Times in L-1210 Leukemic Mice

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Abstract □ Four alkyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol were selected to study the effects of microsomal enzyme induction by phenobarbital on the toxicity changes as reflected by LD_{50} and alteration of survival times in L-1210 leukemic mice. In the phenobarbital pretreated mice the LD_{50} for the ethyl ether of *p*-*N*,*N*-bis(2-chloroethyl)-aminophenol was decreased from 1641 to 1213 μm/kg. This result suggests that *O*-dealkylation is the major metabolic pathway. The LD_{50} for the propyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol was increased by the pretreatment from 605 to 678 μm/kg. The LD_{50} for the butyl ether was increased from 714 to 910 μm/kg. An additional metabolic pathway, (ω-1)-hydroxylation, is suggested for the propyl and butyl ethers. The hexyl ether appeared to be unaffected by the pretreatment; thus, *O*-dealkylation was ruled out as a major pathway. In the survival studies, the pretreatment reduced the antitumor effectiveness of the ethyl and the butyl ethers. The survival times were increased for some dose levels for the propyl ether. No significant trend in survival times was observed for the hexyl ether in the pretreated mice.

Keyphrases □ Microsomal enzyme induction—effects on toxicity in mice, survival times in L-1210 leukemic mice of *p*-*N*,*N*-bis(2-chloroethyl)aminophenyl alkyl ethers, phenobarbital □ *p*-*N*,*N*-Bis(2-chloroethyl)aminophenyl alkyl ethers—effects of microsomal enzyme induction on toxicity in mice, survival in L-1210 leukemic mice, phenobarbital

Variations in the alkyl chain lengths of esters and ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol have been shown to alter the toxicity and survival times of L-1210 leukemic mice over that of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol (1–3). It was proposed that the fatty acid esters would undergo ester hydrolysis and release the *p*-*N*,*N*-bis(2-chloroethyl)aminophenol and its other degradation products. Previous reports suggest that the short chain alkyl aryl ethers may be metabolized by an α-hydroxylation pathway resulting in *O*-dealkylation, while the longer chains may be subjected to another pathway. It is likely that the metabolism of this series of alkyl aryl ethers would be similar to the *p*-nitrophenyl alkyl ethers reported previously (4). The hexyl ether of *p*-nitrophenol was not *O*-dealkylated as were the lower members in that series. Although the metabolism of the hexyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol has not been studied, toxicity studies and survival studies in L-1210 leukemic mice have suggested the absence of the α-hydroxylation pathway. A dual metabolic pathway, α- and (ω-1)-hydroxylation, is possible for the *n*-propyl and *n*-butyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol. These pathways on *p*-nitrophenyl-*n*-butyl ether have been demonstrated (5) with the identification of 3-hydroxy-*n*-butyl ether of *p*-nitrophenol as a major metabolite.

The purpose of this research was to study the effects of microsomal enzyme induction in mice on the toxicity and survival times of L-1210 leukemic mice treated with the ethyl, *n*-propyl, *n*-butyl, and *n*-hexyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol. An increase in toxicity would be expected to accompany an increase in *O*-dealkylation in induced mice and provide further evidence for the presence of the (ω-1)-hydroxylation pathway for the higher members in the alkyl ether series.

EXPERIMENTAL

Test Animals—DBA/2[1], BDF_1[1], and HA/ICR mouse strains[2] and L-1210 leukemic mice (tumor source)[3] were used.

Instruments—The necessary equipment included an electronic cell counter[4], a channelizer[4], a dilutor[4], an *xy* recorder[4], a hemocytometer[5], and a microscope[6].

Materials—Counting diluent[4], red cell-lysing reagent[7], crystal violet[8], Giemsa stain[7], isotonic diluting solution[9], trypan blue[10], and Wrights' strain[8] were used. The alkyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol (the ethyl-propyl-, butyl-, and hexyl ethers) and *p*-*N*,*N*-bis(2-chloroethyl)aminophenol were prepared and have been reported previously (3). All other chemicals and reagents were obtained from commercial sources.

Microsomal Enzyme Induction—A modification of a previous method was employed in the enzyme induction phases of the study (6). Thirty-five HA/ICR mice (6-weeks-old) were given sodium phenobarbital (75 mg/kg) by intraperitoneal injection daily for 7 days. The onset and duration of sleeping time for each mouse was recorded. Sleeping time is the time interval between the loss and recovery of the righting reflex (6). Changes in the duration of sleeping time were used as an indicator of the degree of enzyme induction. After the 7th day of treatment the mice were randomly divided into seven groups, five mice to each. On day 9 sodium phenobarbital (75 mg/kg) was administered to the first group to observe any changes in the onset and duration of sleeping time after the discontinuation of the pretreatment for one day. The same dose of sodium phenobarbital was administered to the remaining groups 2, 3, 4, 5, 6, and 7 days, respectively, after the discontinuation of the daily pretreatment.

Toxicity Evaluation—HA/ICR mice were divided into groups of six for the test and control groups. The test groups were given sodium phenobarbital (75 mg/kg ip) daily for 7 days prior to intraperitoneal injections of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol or its alkyl ethers. Each injection consisted of a known concentration of each drug dissolved in 0.1 ml of propylene glycol. The mice were observed and weighed daily for 28 days. The number of mortalities at each dose level was evaluated by a previous method to determine the LD_{50} number for each drug in the test and control groups (7).

Tumor Transplant Procedure—DBA/2 mice bearing L-1210 leukemia for 7 days were used as a source for tumor cells. The L-1210 cells were collected according to a previously described procedure (1). Inoculations containing 10^5 L-1210 cells were administered intraperitoneally to groups of BDF_1 mice to be used in the survival studies.

[1] Jackson Laboratories.
[2] ARS/Sprague-Dawley.
[3] National Cancer Institute.
[4] Model ZB Counter and Accessories, Coulter Electronics.
[5] American Optical Co.
[6] Model RA, Carl Zeiss, West Germany.
[7] Fisher Scientific Co.
[8] Matheson, Coleman, and Bell.
[9] Microbiological Associates.
[10] Allied Chemical Co.

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Survival Studies—One group of healthy BDF_1 mice was pretreated with sodium phenobarbital (75 mg/kg) daily for 5 days. The alkyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol were administered intraperitoneally in propylene glycol to two groups of leukemic mice, one group referred to as the control, which did not receive the phenobarbital pretreatment, and the group described above. Dosages of 25, 50, 75, 100, 150, and 200 mg/kg of the alkyl ethers were given on days 2 and 5 after inoculation to subgroups of six mice for the control group and the pretreated group. An additional group of six BDF_1 mice inoculated with L-1210 cells was employed as the untreated control and received only the injection vehicle, propylene glycol.

RESULTS AND DISCUSSION

The onset and duration of sleeping times for mice pretreated with phenobarbital are reported in Table I and Fig. 1. The onset time for sleeping did not show any relationship with the degree of enzyme induction. The sleeping times gradually decreased during the periods of daily injections of phenobarbital, but gradually returned to normal after termination of the treatment. The increased function of the microsomal enzyme system persisted into the following week. Hypertrophy of the mice livers was observed in all studies 3 or 4 days after the pretreatment was initiated and disappeared after the daily injections were stopped. This observation is consistent with a previous report (8).

In the toxicity studies on the nitrogen mustards, a 7-day pretreatment period was considered adequate for the induction period. Table II shows the results of the toxicity studies performed on the five compounds in phenobarbital pretreated mice and untreated mice. The LD_{50} for compound I, *p*-*N*,*N*-bis(2-chloroethyl)aminophenol, was 164 μm/kg (38 mg/kg) in untreated HA/ICR mice. In the phenobarbital pretreated mice, a reduction in toxicity was suggested by the increase in the LD_{50} to 253 μm/kg (59 mg/kg). This increase in LD_{50} was probably due to an increase in the rate of conjugation of compound I, which should have resulted in a faster excretion rate (9).

A tenfold decrease in toxicity was observed for compound II, the ethyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol, over that of the parent compound (I) in untreated mice. The LD_{50} was 1641 μm/kg which decreased to 1213 μm/kg when the mice were pretreated with phenobarbital. It is presumed that compound II is *O*-dealkylated to the more toxic mustard, compound I, which would lead to one of the ultimate metabolites through hydrolysis of the chloroethyl groups. Some of the toxicity expected from the *O*-dealkylation pathway *via* hydroxylation on the α-carbon on the alkyl group would be mollified by an increase in the rate of conjugation of compound I, its metabolites, other metabolites which have hydroxyl groups, and the various rate processes of the α- and (ω-1)-hydroxylation pathways.

The toxicity of compound III was greater than compound II but less than compound I. The LD_{50} of compound III was 605 μm/kg (160 mg/kg) in untreated mice and increased to 678 μm/kg in the pretreated mice. Compound III, the propyl ether, could undergo metabolism by the α-hydroxylation and the (ω-1)-hydroxylation pathways. If the α-hydroxylation were the major pathway, the toxicity for the propyl ether would be expected to increase in the pretreated mice due to the increased formation of the parent compound, *p*-*N*,*N*-bis(2-chloroethyl)aminophenol. The (ω-1)-hydroxylation of compound III would lead to the 2-hydroxy propyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol which should be less lipid soluble and possibly less toxic.

Similar results were obtained for compound IV. The LD_{50} was 714 μm/kg (207 mg/kg) in untreated mice and increased to 910 μm/kg in the pretreated mice.

Both hydroxylation pathways would be expected for the butyl ether, but α-hydroxylation would have to play a minor role to explain the lowered toxicity in the pretreated mice. If the butyl ether mustard is metabolized by the (ω-1)-hydroxylation pathway, it would appear as the 3-hydroxybutyl ether mustard or corresponding metabolites. This pathway was verified for *p*-nitrophenyl butyl ether (5). The increase in the LD_{50} for compound IV suggests that phenobarbital pretreatment enhances the rate for the alternate pathway over that of α-hydroxylation.

The effect of pretreatment on the toxicity of compound V was negligible. The LD_{50} was 1069 μm/kg (340 mg/kg) in the untreated mice and changed only slightly to 1104 μm/kg in the pretreated mice. This result was anticipated since the aryl alkyl ether mustards would be expected to follow the same metabolic pathways as reported for the alkyl ethers of *p*-nitrophenol (4). In that report *O*-dealkylation was negligible for the hexyl ether of *p*-nitrophenol. The insignificance of the α-hydroxylation pathway in mice is supported by the results of the toxicity studies.

The expected metabolite of compound I, *p*-*N*,*N*-bis(2-hydroxyethyl)aminophenol, has been isolated from rat blood as a major metabolite of compound II and to a lesser extent for compounds III and IV. Unidentified metabolites which did not conform to the α-hydroxylation pathway were observed for compounds III and IV[11].

The results of a study of the influence of microsomal enzyme induction on the survival times of leukemic mice treated with alkyl ethers of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol are presented in Table III and illustrated in Fig. 2. The ethyl ether, compound II, would be expected to undergo α-hydroxylation and hydrolysis would yield acetaldehyde and the more toxic form of the mustard, compound I, and its metabolites. The ethyl mustard produced a maximum survival of 126% T/C at a dose of 383 μm/kg in the control group (leukemic mice treated with the mustard

Table I—Onset and Sleeping Duration for Mice Pretreated with 75 mg/kg of Sodium Phenobarbital[a]

Days	Dosing Omitted on Days	Onset, Minutes (*SE*)[b]	Sleeping Time, Minutes (*SE*)[b]
1	—	23.2(±0.46)	94.2(±0.57)
2	—	20.2(±0.80)	77.4(±2.09)
3	—	19.6(±0.96)	74.3(±1.48)
4	—	18.1(±0.65)	70.6(±1.46)
5	—	18.3(±0.61)	62.9(±0.59)
6	—	21.0(±1.10)	47.4(±1.33)
7	—	19.9(±0.92)	43.9(±1.01)
8	—	—	—
9	8	22.0(±2.00)	43.0(±3.54)
10	8–9	19.6(±1.63)	48.0(±4.90)
11	8–10	23.0(±1.22)	49.0(±4.58)
12	8–11	22.0(±2.55)	55.0(±3.16)
13	8–12	20.0(±4.25)	62.0(±4.90)
14	8–13	17.0(±1.22)	64.0(±5.09)
15	8–14	25.4(±2.38)	67.0(±2.65)

[a] Administered intraperitoneally in sterile water for injection. [b] Standard error.

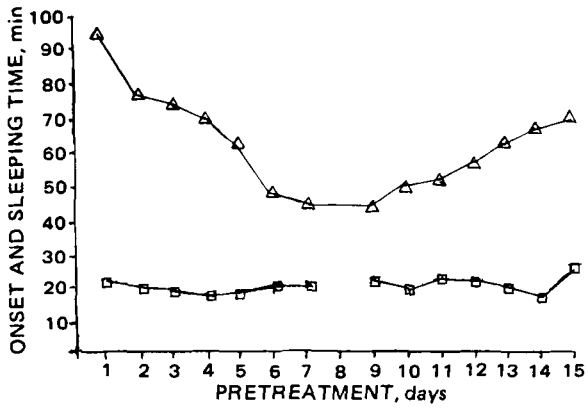

Figure 1—*The sleeping time of mice pretreated with sodium phenobarbital. Key: (□) onset of sleep; (△) sleeping time.*

Table II—Toxicity of *p*-*N*,*N*-Bis(2-chloroethyl)aminophenol and its Alkyl Ethers in Mice Pretreated with Phenobarbital

Compound	Untreated Mice, LD_{50} μm/kg	Untreated Mice, LD_{50} mg/kg	Pretreated Mice LD_{50} μm/kg	Pretreated Mice LD_{50} mg/kg
I (Parent compound)	164	38	253	59
II Ethyl ether	1641	430	1213	318
III Propyl ether	605	167	678	187
IV Butyl ether	714	207	910	264
V Hexyl ether	1069	340	1104	351

[11] Unpublished data.

Table III—Survival Times of L-1210 Leukemic Mice [a] Treated with *p*-*N*,*N*-Bis(2-chloroethyl)aminophenyl Alkyl Ethers and Pretreated with Phenobarbital

Derivative Compound	Dose, μm/kg	Untreated Control Mean Survival Days (±*SE*)	Control Mean Survival Days (±*SE*)	T/C %[b]	Phenobarbital Pretreated Mean Survival Days (±*SE*)	T/C %
II	96	8.50(0.34)	9.83(0.31)	117	8.33(0.21)	98
Ethyl ether	192	8.50(0.34)	10.00(0.68)	118	8.67(0.33)	102
	287	8.50(0.34)	9.33(0.56)	110	9.33(0.56)	110
	383	8.50(0.34)	10.67(0.21)	126	9.00(0.97)	106
	479	8.50(0.34)	10.17(0.17)	120	10.16(0.47)	120
	575	9.00(0.37)	9.37(0.92)	104	7.50(1.18)	84
	766	9.00(0.37)	7.33(0.67)	81	6.00(0.82)	67
III	91	8.50(0.34)	10.33(0.33)	122	9.33(0.21)	110
Propyl ether	181	8.50(0.34)	10.83(0.48)	127	11.00(0.52)	129
	273	8.50(0.34)	9.66(0.95)	114	10.67(0.42)	126
	364	8.50(0.34)	10.33(0.56)	117	12.33(0.33)	145[c]
	455	8.50(0.34)	10.00(1.10)	118	10.50(1.06)	124
	546	8.50(0.34)	9.16(0.87)	108	8.17(0.60)	96
	727	9.00(0.37)	9.50(0.34)	106	8.67(0.76)	96
IV	87	9.00(0.37)	9.50(0.34)	106	8.67(0.76)	96
Butyl ether	173	9.00(0.37)	10.00(0.63)	111	9.50(0.22)	106
	260	9.00(0.37)	10.33(1.15)	115	9.00(0.86)	100
	346	9.00(0.37)	10.33(1.36)	115	7.83(0.98)	87
	519	9.00(0.37)	12.50(0.56)	139	7.50(0.81)	83[c]
	692	9.00(0.37)	12.83(0.54)	143	10.17(1.70)	113
	727	9.00(0.37)	6.50(0.34)	72	6.33(0.42)	70
V	0	9.33(0.51)	—	—	9.33(0.51)	100
Hexyl ether	17	9.33(0.51)	10.00(0.37)	107	10.67(0.42)	114
	33	9.33(0.51)	10.17(0.31)	109	9.83(0.31)	105
	83	9.33(0.51)	10.00(0.52)	107	9.67(0.49)	104
	166	9.33(0.51)	10.00(0.45)	107	10.00(0.45)	107
	249	9.33(0.51)	10.50(0.43)	113	10.67(0.42)	114
	332	9.33(0.51)	10.17(0.48)	109	9.33(0.71)	100
	415	9.33(0.51)	10.67(0.42)	114	10.17(0.83)	109
	498	9.33(0.51)	9.33(0.33)	100	8.33(0.56)	89
	645	9.33(0.51)	9.83(0.83)	105	9.50(0.85)	102

[a] Six BDF_1 mice were used for each dose level for each group. [b] Statistical differences at $p < 0.05$ for Control *versus* Pretreatment. [c] The T/C % represents the rates of the sum of days (T) each treated animal survives to the sum of the number of days each control animal (C) survives times 100.

only), but was reduced to 106% T/C in the corresponding group pretreated with phenobarbital. The survival times for all dose levels of compound II were lower in the phenobarbital pretreated groups over the control groups, with the exception of two dose levels. A dose level of 479 μm/kg produced a survival of 120% T/C in both groups. It was generally expected that an increase in the rate of *O*-dealkylation of compound II in the pretreated mice would increase host toxicity and overcome some of the benefits of drug treatment.

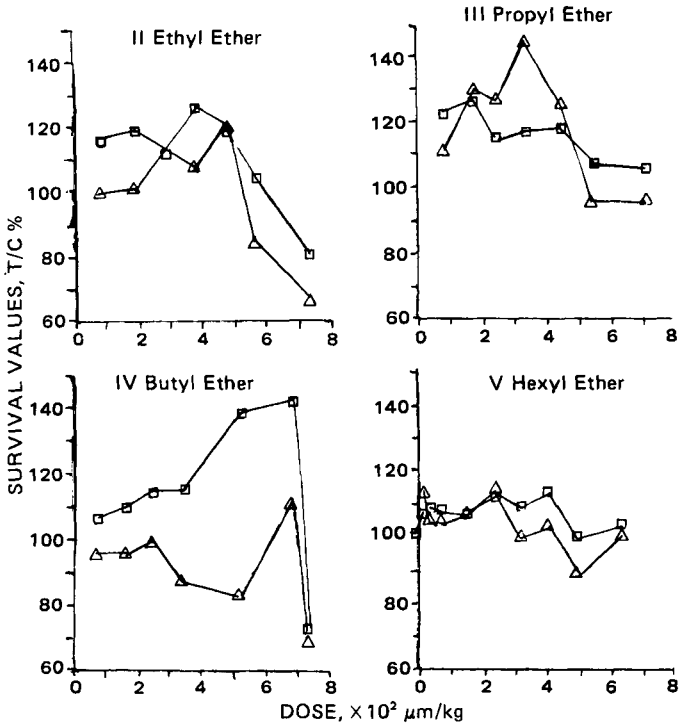

Figure 2—*Survival values of L-1210 leukemic mice treated with* p-N,N-*bis(2-chloroethyl)aminphenyl alkyl ethers, (II) ethyl ether, (III) propyl ether, (IV) butyl ether, and (V) hexyl ether. Key: (□) Control; (△) Pretreated.*

The propyl ether, compound III, was more effective in increasing survival values at the four dose levels ranging from 181 through 455 μm/kg in the pretreated groups. The highest survival value was 145% T/C in the pretreated group, which was significantly different from the control value of 117% T/C. If the metabolism of compound III were accelerated by the α-hydroxylation pathway in the pretreated group, a decrease in the survival value would have been expected, as observed with compound II, due to increased host toxicity. α-Hydroxylation has been demonstrated in propyl ethers (4), but an alternate pathway would be suspected to satisfactorily explain these results. One other major metabolite, which would result from an (ω-1)-hydroxylation, has been suggested (5). An active metabolite other than the parent compound may be the 2-hydroxy propyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol. Although the differences are not significant, in the lowest dose, 91 μm/kg, and the two highest doses, 546 and 727 μm/kg, the survival values of the pretreated groups fell below the untreated groups.

All dose levels of compound IV exhibited lower survival values in the pretreated groups when compared with the control groups. A dose level of 519 μm/kg produced a significant difference between survival values of pretreated *versus* control groups, although the best value, 143% T/C, occurred at 692 μm/kg in the control group. A decrease in the rate of *O*-dealkylation of *p*-nitrophenyl alkyl ethers as the length of the carbon chain increased has been found (4). The (ω-1)-hydroxylation pathway is favored as a possible explanation consistent with the results from both the toxicity and the survival data. If the 3-hydroxybutyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol is produced more rapidly than the parent compound, that would provide a possible explanation for the toxicity differences between control and pretreated group, and it would have less antitumor activity than compound IV.

The results from previous studies on the *O*-dealkylation of *p*-nitrophenyl alkyl ethers and survival studies on the *p*-*N*,*N*-bis(2-chloroethyl)alkyl ethers are consistent with the lack of significant differences between the control groups and pretreated groups for the hexyl ether. Since α-hydroxylation of hexyl aryl ethers is unlikely, minimal effects

Table IV—Statistical Analysis of the Survival Studies on an SPSS Multiple Regression Subprogram

Drug	Dose Range	R^2 [a]	a [b]	b [b]	c [b]	$B(b)$ [c]	$B(c)$ [c]	$F(b)$ [d]	$F(c)$ [d]
Ethyl ether	25–100	0.183	8.982	0.0137	−0.995	0.377	−0.365	8.3	7.8
	125–200	0.387	15.714	−0.0463	NS[e]	−0.623	NS	22.62	NS
Propyl ether	25–100	0.103	9.677	0.0155	NS	0.3219	NS	4.950	NS
	125–200	0.349	15.262	−0.0433	NS	−0.591	NS	19.365	NS
Butyl ether	25–100	0.268	8.556	0.0333	NS	0.518	NS	5.854	NS
	25–150	0.237	9.683	0.0138	−2.189	0.235	−0.433	4.993	16.96
Hexyl ether	25–200	0.0221	10.21	−0.0028	NS	−0.149	NS	2.391	NS

[a] R^2 (coefficient of determination) indicates the proportion of variation in life span explained by dose only or by both dose and treatment. [b] The form of multiple regression equation: $Y = a + b(\text{dose}) + c(\text{treatment})$ Y = life span, a = intercept of y axis, b = regression coefficient for dose, c = regression coefficient for treatment (dummy variable). [c] $B(b)$ indicates the number of standard derivation units of change in life span that could be predicted when dose changes by one standard unit. $B(c)$ indicates the number of standard derivation units of change in life span that could be predicted when treatment changes by one standard unit. [d] $F(b)$ presents the F value of dose. $F(c)$ presents the F value of treatment. Except $F(b)$ of hexyl ether, all other F values are greater than the critical values at the 0.05 level of significance. [e] NS = no significance.

on toxicity or survival times in leukemic mice would be expected to result from phenobarbital pretreatment (3, 4). It is not known if the hexyl ether of *p*-*N*,*N*-bis(2-chloroethyl)aminophenol undergoes (ω-1)-hydroxylation, but if it were to occur, these results suggest a metabolite similar in toxicity and antitumor activity to compound V.

A regression analysis was performed on survival data from the control and pretreated groups (Table IV). The analysis, taken over the entire range of doses, failed to show significant trends for any of the compounds tested. Negative trends in the ascending points for data sets from compounds II and IV indicate a decrease in the antitumor effectiveness for the pretreated mice. Crossover trends at the lowest and two highest doses for compound III precluded significance. The medium level doses were not tested. No trend could be established for compound V.

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Antipyrine and Acetaminophen Kinetics in the Rat: Comparison of Data Based on Blood Samples from the Cut Tail and a Cannulated Femoral Artery

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Abstract □ Antipyrine and acetaminophen kinetics were determined from concentration data obtained by simultaneous blood sampling from the cut end of the tail and a cannulated femoral artery in the rat. Significant differences in concentrations and kinetics for both drugs were found by comparison of the two sampling sites. The hypothesis that the differences were due to a low tail blood flow was tested. The tail blood flow was measured with a microsphere technique, and tail antipyrine concentrations were calculated from the relationship between arterial antipyrine concentration, tail flow, and time for comparison with the observed antipyrine concentrations. Mean blood flow of the rat tail was 0.02 ml/min/ml tail tissue at 22°, which was 8.8 and 0.9% of the liver and kidney flow, respectively. Tail flow increased more than twofold by elevation of the tail temperature to 37°. The calculated tail antipyrine concentration *versus* time curve showed a very close correspondence to the observed antipyrine tail concentration *versus* time curves. The results show that tail flow is a major determinant of antipyrine tail concentration in the rat. Kinetic data based on blood samples from the cut end of the tail, therefore, should be interpreted with caution.

Keyphrases □ Antipyrine—pharmacokinetics in the rat, comparison of data based on blood samples from the cut tail and a cannulated femoral artery □ Acetaminophen—pharmacokinetics in the rat, comparison of data based on blood samples from the cut tail and a cannulated femoral artery □ Pharmacokinetics—acetaminophen and antipyrine in the rat, comparison of data based on blood samples from the cut tail and a cannulated femoral artery □ Microsphere technique—pharmacokinetics in the rat, comparison of data based on blood samples from the cut tail and a cannulated femoral artery

The development in recent years of a multitude of sensitive drug assays in microsamples of blood and a variety of techniques for repeated blood sampling have made it possible to perform pharmacokinetic studies in individual animals as small as mice, rats, and guinea pigs. Whereas published methods for drug assays are routinely validated

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