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PHYSIOLOGICAL DISPOSITION AND BIOCHEMICAL REACTIONS OF THE ^{14}C-4′,4″-*BIS*(2-IMIDAZOLIN-2-YL)2-CHLOROTEREPHTHALANILIDE DIHYDROCHLORIDE IN MICE AND RATS *IN VIVO* AND *IN VITRO**

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Abstract—Quantitative drug distribution studies using ^{14}C-labeled NSC 38280 in mice after intravenous administration demonstrated the greatest accumulation of the substance in liver and kidneys. The blood showed much lower levels, with a half-life of approximately 30 min after injection. After intraperitoneal administration, the tissues that accumulated most of the substance were the same as after i.v. injection, but the blood contained only about one-third of the maximal concentration measured after i.v. injection. After both i.v. and i.p. injection, liver and kidneys showed a high retention of the substance over the 5 days of the experiment. Considerable accumulation was also found in the adrenals after both routes of administration.

After i.v. injection into BDF_1 mice with advanced P-388 leukemia, significant activities were found in the leukemic ascitic fluid which remained at the same level even when the blood levels of the same mice fell to insignificant activity.

The excretion in the bile of rats was about 10 per cent within 24 hr, and in the feces of intact rats it was approximately 16 per cent. The excretion in the urine of intact rats in 24 hr was about 21 per cent. In 72 hr, urine and feces contained 46 per cent of the injected activity.

Studies *in vivo* and *in vitro* have showed so far no enzymatic cleavage of the amide linkage of the compound, either by enzymes of the gastrointestinal tract or by leukemic ascitic fluid from mice whose leukemias were sensitive (P-815 and P-388) or resistant (P-815/38280, P-388/38280) to NSC 38280.

THE GREAT interest that the group of terephthalanilides[1] has aroused in experimental cancer[2–11] and in trial in patients with acute leukemia[12, 13] has made studies of absorption, distribution, and elimination in mice and rats desirable.

The availability of the ^{14}C-labeled 4′, 4″-*bis* (2-imidazolin-2-yl)2-chloroterephthalanilide (NSC 38280)† made its localization and quantitative estimation easy. The

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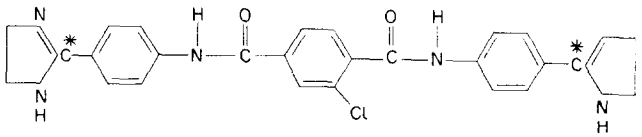
labeling was effected in the 2-position of the imidazolin ring (^{14}C marked with *) and seemed to be very stable, since no activity was found in the expired CO_2 in two experiments.



MATERIALS AND METHODS

Mouse experiments

A stock solution containing 0·109 mg of the ^{14}C-labeled compound (specific activity: 2·50 mc/mM)/ml of 5% glucose in water was injected intravenously or intraperitoneally. BDF_1 mice having an average weight of 22 g were used in these experiments. The dosage administered for i.v. and i.p. distribution was 4·50 mg/kg. In the accumulation experiments and in the experiments with mice inoculated with leukemia P-388 the administered dose was 2·5 mg/kg i.p.

In the distribution experiments, after intervals of 5 min and 1, 4, 8, 24, 48, and 120 hr, the mice were exsanguinated by heart puncture under ether anesthesia. The different organs were dissected, washed in distilled water, and 50 to 250 mg (wet weight) weighed out on ashless filter paper. Blood and ascitic fluid were applied directly on an especially thick, ashless filter paper.[14] Samples were then dried overnight in a vacuum desiccator. Combustion analysis of tissue and paper, using the technique of Kalberer and Rutschmann,[14] was made afterward and the samples counted on a Packard Tri-Carb scintillation counter, model 314 EX. The activity was expressed as the factor F:[15]

$$F = \frac{\text{specific activity of tissue} \times \text{body weight}}{\text{total activity administered}}$$

Rat experiments

The same stock solution as above was used in the excretion and respiration experiments. Healthy white male Wistar rats of an average weight of 180 g were used. An amount of 1·2 ml of the stock solution was administered to each rat either intravenously or per os, which represented a dosage of 0·73 mg/kg. The pooled urines and feces of two animals were collected in a modified metabolism cage described by Brittain.[16]

In experiments designed to study the biliary excretion of the drug, the bile duct was cannulated under ether anesthesia with a polyethylene catheter, and the rat was supported in an especially constructed frame. After closing the abdominal wall the bile was collected in fractions in volumetric flasks without anesthesia. Aliquots of the diluted samples were analyzed as described under distribution experiments.

In the respiration experiments the dried expired air was collected in fractions every 2 hr in ethanolamine in ethylene glycolmonomethylether, 1:2 v/v. An aliquot of 10 ml of the mixture toluene:ethylene glycolmonomethylether, 2:1 v/v, containing 0·55% diphenyloxazol,[17] was added and measured in the scintillation counter.

Enzymatic studies

For the determination of a possible hydrolysis of the anilide linkage the stomachs and intestinal tracts were removed from two white male rats. The two unwashed

stomachs and contents were pooled and homogenized in a Waring blender with 5 ml 5% glucose in water. The unwashed intestines and contents were also pooled and homogenized in 5 ml. Each homogenate was divided into two parts (approximately 5 ml) and incubated for 1 or 12 hr at 37° with 4 mg NSC 38280 in 1 ml distilled water. No attempt was made to buffer these breis, and pH determinations were not made. After filtration through a layer of Hyflow Super-cel the color reaction, as described by Rogers and York,[18] with and without hydrolysis, was studied. It was postulated that, if an enzymatic cleavage of the linkage occurred during incubation, a color should develop without previous chemical hydrolysis, thus indicating a free primary aromatic amino group. In addition, the incubation filtrate was concentrated and compared on thin-layer plates with the original compound.

System: n-butanol:acetic acid:water, 4:1:5. Gel: silica gel G (Merck, Germany). Identification of the spots: Dragendorff's reagent for inactive material. Radioactive spots were localized with a Baird-Atomic scanner assembly, model RSC-5B.

Ascitic fluids from mice with leukemia P-815 and with a subline of P-815, made resistant to NSC 38280 (P-815/38280), were treated as the homogenates of the gastrointestinal tracts. Intraperitoneal administration of NSC 38280 *in vivo* in a dose of 16 mg/kg to P-815, P-388, P-815/38280, and P-388/38280 mice was also studied; 1 and 4 hr after injection, alcohol extracts of their ascitic fluids were subjected to the same colorimetric and thin-layer chromatographic analyses as above.

RESULTS AND DISCUSSION

A. Experiments with mice

1. Five min after a single i.v. injection the highest F value (7·22) was found in the lungs (Fig. 1) of BDF_1 mice. This can be explained by their anatomical position in the

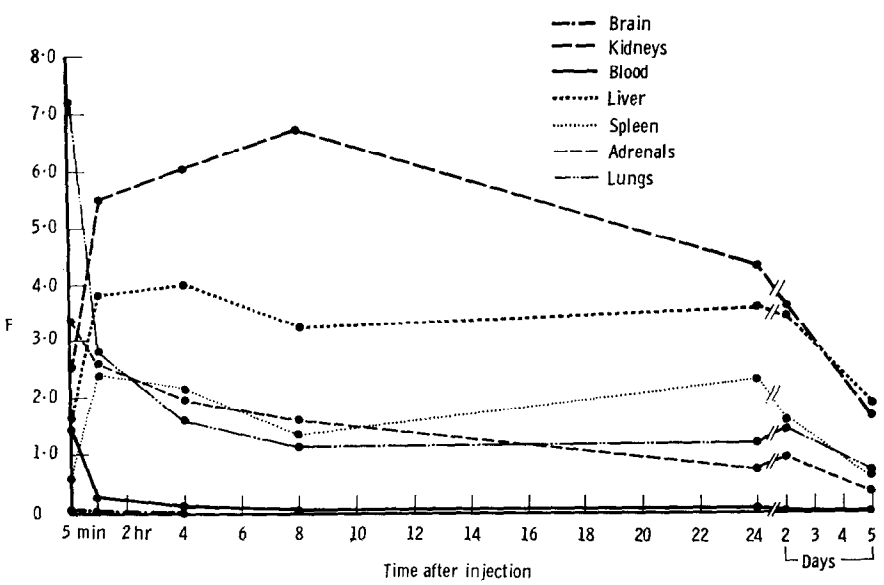


FIG. 1. Distribution of NSC 38280 in mice; intravenous administration; single dose 4·5 mg/kg.

blood circulation. Similar results have been shown with other substances—e.g. the large group of phenothiazines.[19, 20] The decrease of the radioactivity was much more rapid in the lungs than in the liver and kidneys, where high F values were also found. Although the F values were initially higher in the kidneys, the longest retention was found in the liver, where 90 per cent of the highest activity ($F = 4{\cdot}03$) could still be demonstrated after 24 hr ($F = 3{\cdot}61$), and only little less than 50 per cent after 5 days ($F = 1{\cdot}92$). In the kidneys, with a high of $F = 6{\cdot}71$ at 8 hr, the corresponding values were 65 per cent and about 25 per cent respectively. These high storage rates in liver and kidneys correspond to the very slow excretion rates in the rat experiments. Similar findings are known for phenanthridinium derivatives[21] and for Stilbamidine.[22] The F values in the blood showed, after an initial 5-min value of 1·45, a very rapid fall and a half-life of about 30 min. This short half-life of the compound in blood is presumably due to the strong affinity of the liver and kidneys for the substance and only to a small extent to the excretion through bile and urine. The extremely low values for the whole brain could be due to its blood content rather than to a passage through the blood–brain barrier.

After a single i.p. administration (Fig. 2) the highest activity was found in the kidneys 4 hr after the injection ($F = 7{\cdot}69$), followed by the liver ($F = 4{\cdot}32$ after 8 hr)

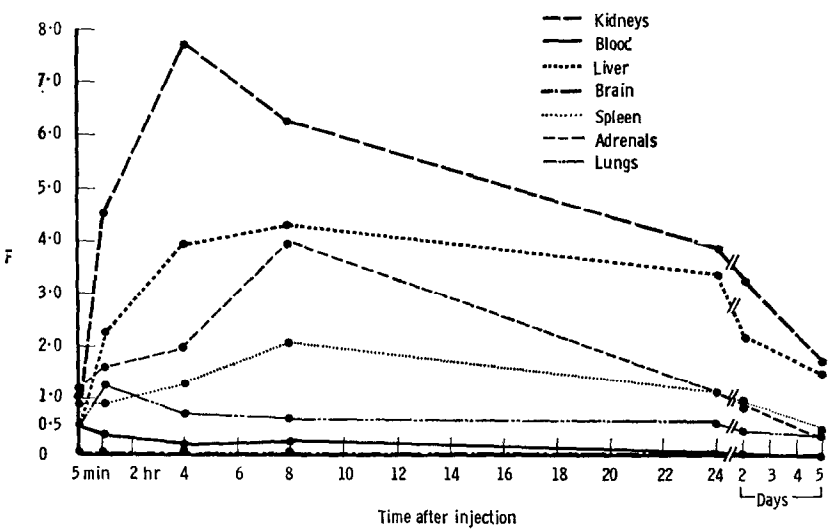


FIG. 2. Distribution of NSC 38280 in mice; intraperitoneal administration; single dose 4·5 mg/kg

and the adrenals ($F = 4{\cdot}00$ after 8 hr). Five min after the i.p. injection, the blood showed only about one-third of the corresponding value after i.v. injection ($F = 0{\cdot}46$), but decreased in about the same way as after i.v. injection. Slightly higher F values were found after i.p. injection in the intestines, compared with those after i.v. injection. This may have been due to local adsorption to the serosa despite careful washing in saline. Intermediary increase of F values in the i.v. experiments after 8 to 24 hr (Fig. 1) in blood, spleen, and liver may indicate a possible small reabsorption of the unchanged compound, metabolites, or split products of the original terephthalanilide.

2. In the cumulative experiment, during which 12 i.p. injections were administered over 13 days (Table 1), marked accumulation in the kidneys and liver, and to a lesser extent in the adrenals, spleen, intestines, and lungs, was found. In blood and brain, however, no significant accumulation was demonstrated. The accumulation in liver, kidneys, spleen, and lungs might explain some of the toxic and pathological symptoms found in rats and dogs after single and repeated i.v. administration, such as weight loss, electrolyte disturbances, and congested, edematous lungs, kidneys, and liver.[8]

TABLE 1. CUMULATIVE ADMINISTRATION OF ^{14}C-NSC 38280 IN MICE

Daily intraperitoneally, 2·5 mg/kg; numbers indicate the counts per minute per 100 mg of wet tissue.

Day of exp.	Day sample taken	No. inj.	Blood	Brain	Lungs	Intestines	Spleen	Liver	Kidneys	Adrenals
1										
2	2	1	9	3	170	530	569	1110	2073	685
3	3	2	10	3	454	640	1023	2737	3716	1107
4	4	3	19	8	859	1032	1512	3878	6430	1431
5		4								
6	6	5	36	23	1657	1799	2200	6230	8885	2161
7		6								
8	8	7	16	22	1460	1718	3783	6353	7484	3303
9		8								
10	10	9	23	23	1751	2333	3825	8170	12,034	3517
11		10								
12		0								
13		11								
14	14	12	22	30	2299	2771	3468	5480	10,524	6192

TABLE 2. *F* VALUES IN BLOOD AND ASCITES OF MICE AFTER INTRAVENOUS AND INTRAPERITONEAL ADMINISTRATION OF ^{14}C-NSC 38280

Time after injection (hr)	Injection i.v.		Injection i.p.	
	Ascites	Blood	Ascites	Blood
1	0·34	0·43	2·33	0·31
4	0·32	0·09	2·80	0·05
24	0·45	0·06	3·17	0·02

3. When the compound was injected i.v. or i.p. in BDF_1 mice with advanced P-388 leukemia, the blood levels were comparable to those found in healthy BDF_1 mice. Of special interest, however, was the activity of the ascitic fluid which showed, in contrast to the blood levels, a retention or perhaps even an accumulation of radioactivity over 24 hr (Table 2). The relatively high activity of the ascitic fluid after i.v. injection suggested a strong diffusion or an active secretion into the abdominal cavity. The rapid fall-off and the comparatively low activity in the blood at 24 hr after i.v. injection makes the retention in the ascitic fluid even more interesting. When the activities in liver and spleen are compared in normal mice and mice with P-388 leukemia after i.p. injection, the peak *F* value in the leukemic mice is only about half that found in healthy mice. In contrast, after i.v. injection there were no significant differences between leukemic and normal mice in these levels. This suggests that, after i.p. administration,

either a diminished absorption through the infiltrated peritoneum or a local absorption and retention of the substance by the leukemic cells occurred.

B. Experiments with rats

Bile experiments. When the drug was injected i.v., the excretion in the bile of rats (Table 3) was relatively small (10·5 per cent of the injected amount during the first 24 hr). This corresponded well with the small loss of activity in the liver of mice during the first 24 hr. After 24 hr the excretion in the bile still continued but to a lesser degree. The excretion in the urine of the same rats showed an average of 15 per cent in 24 hr, which was about half the loss of activity in the kidneys of mice during the same time.

TABLE 3. ADDITIVE ACTIVITY IN BILE AND URINE OF RATS IN PER CENT OF INJECTED ACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF ^{14}C-NSC 38280

	Experiment 1					Experiment 2				
	Bile		Urine			Bile		Urine		
Time aft. inj. (hr)	% Act. excreted in interv.	% Act. excreted additive	% Act. excreted in interv.	% Act. excreted additive	Bile & urine % excreted additive	% Act. excreted in interv.	% Act. excreted additive	% Act. excreted in interval	% Act. excreted additive	Bile & urine % excreted additive
2	0·66	0·66				2·88	2·88			
4	1·96	2·62				1·65	4·53			
8·25						4·88	9·41	18·15	18·15	27·56
9	3·54	6·16	9·35	9·35	15·51					
24	4·16	10·32	2·00	11·35	21·67	1·28	10·69	0·54	18·69	29·38
26						0·22	10·91			

TABLE 4. URINE AND FECES EXCRETION OF INTACT RATS IN PER CENT OF THE INJECTED ACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF ^{14}C-NSC 38280

Time after inject. (hr)	Urine		Feces		Urine + feces
	% Activ. excreted	Additive % excreted	% Activ. excreted	Additive % excreted	Additive %
2	4·13	4·13			
4	6·55	10·68			
9·30	8·20	18·88			
21	1·98	20·86	15·58	15·58	36·44
24	0·29	21·15	0·08	15·66	36·81
48	0·95	22·10	6·25	21·91	44·01
72	0·35	22·45	1·93	23·85	46·30

Intact animals. Similar results were found in the intact rat. The pooled urine and feces of two animals showed the following radioactivity (Table 4). About 21 per cent of the amount injected i.v. was excreted during the first 24 hr in the urine, whereas the feces contained about 16 per cent of the activity over the same interval. After this time the excretion in the feces continued, whereas the amount in the urine fell rapidly. After 72 hr 46·5 per cent of the administered activity had been excreted in feces and urine. These findings correspond to the high activities still remaining in the kidneys and livers of the mice after 48 and 120 hr.

The poor oral absorption of NSC 38280 was indicated by the fact that only 0·37 per cent of the total activity administered was excreted into the urine. If one postulates that the 21 per cent urinary excretion after i.v. dosage corresponds to an absorption of 100%, then the 0·37% urinary excretion after oral administration would correspond to an absorption of about 2%. This very poor absorption explains the ineffectiveness of the compound by the oral route against mouse leukemia.

C. Enzymatic studies

In an attempt to study the stability of the amide linkage toward enzymes, studies were conducted *in vivo* and *in vitro* with the enzymes of the gastrointestinal tract of rats. So far, no indication for such a cleavage of this linkage has been found. The experiments as described in Materials and Methods showed that no color could be obtained without chemical hydrolysis of the incubated reaction mixture, which means that no free primary aromatic amino group could be identified, whereas after hydrolysis with 20% hydrochloric acid the typical diazotized derivative of the added compound was found. The reason for this might be in a steric hindrance by the relatively bulky groups on both sides of the —CO—NH— linkage.

This same question seemed to be of even more importance in the following findings. Recently, certain compounds related to NSC 38280, such as 1,1′-*m*-phenylene *bis* [3-(*p*-2-imidazolin-2-yl-phenyl)urea]dihydrochloride (NSC 57142); 1,1′-*p*-phenylene *bis*[3-(*m*-2-imidazolin-2-yl-phenyl)urea]dihydrochloride (NSC 57143); and N,N″-*bis*[*p*-(N′-methylamidino)phenyl]terephthalamidine, tetrahydrochloride (NSC 57155) have become available. These differ from the original terephthalanilides in having ureido groups instead of the amide linkages (NSC 57142 and NSC 57143) and a meta-substituted central benzene ring (NSC 57142) instead of the para-substituted one of NSC 38280 and no substitutions in the central benzene ring (NSC 57142, NSC 57143 and NSC 57155). NSC 57155 showed even less relation by having amidino groups in place of the amide linkage on both sides of the central benzene ring and methylamidino groups instead of the imidazolin rings in the NSC 38280. These three compounds were active against the sublines of both leukemias P-388 and P-815 made resistant to NSC 38280.[10] Compared with NSC 38280, these three compounds have in common the loss of the amide linkage on either side of the central ring. This suggested that the mechanism of resistance in these leukemias against NSC 38280 might be due to an activation or development of an amidase that would cleave and therefore inactivate NSC 38280. The same colorimetric tests as described above for the experiments with homogenized gastrointestinal tracts also showed no cleavage of the —CO—NH— linkage in this experiment. The lyophilized incubation filtrate showed the same Rf value (0·2) as the original compound. If any enzymatic cleavage had been achieved, a change in the Rf value would have easily been identified, since the Rf value of the resulting *p*-aminophenylimidazolin showed a distinct difference (0·3) in the system used. However, so far, our experiments have shown no such difference in the metabolism of the resistant lines of ascites cells of leukemia P-388 and P-815. Studies *in vivo* and *in vitro* of the metabolism of this compound are in progress.

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