Cancer Chemother. Pharmacol. 3, 171–176 (1979)

Cancer Chemotherapy and Pharmacology

Disposition and Metabolism of Bruceantin in the Mouse*

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Summary. *A microbiologic assay of the agar diffusion type, employing a strain of yeast tentatively identified as Candida macedoniensis, was developed to study the disposition, excretion, and metabolism of the antitumor agent bruceantin (BN; NSC 165563) in the mouse. Bioautographic studies showed the assay to be specific for BN. Normal and tumor-bearing male BDFl mice were studied. Tumor-bearing mice were implanted sc with 10^6 L1210 ascites cells and held for 7 days prior to dosing. Average tumor weight was 307 mg and advanced generalized disease was evident. Three groups of 5 or 10 mice were injected iv with BN (1.5 mg/kg; approx. LD_{10}). At various times after injection, blood, tissues, urine, and feces were obtained and extracted with chloroform to recover BN. Recovery of BN was in the range of 91 to 121%. Disposition and excretion of BN were similar for normal and tumor-bearing mice. Decay of BN in blood was biphasic with α-phase half-lives of 5 to 6 min. Estimated half-lives for the β-phase were possibly > 0.5 day. Average zero time intercepts for α- and β-phases were 550 and 51 ng/ml respectively. Higher levels of BN were found in lung, pancreas, intestine, and spleen (1–6 μg/g) than in liver, kidney, and tumor (0.3–0.5 μg/g) after 15 min. Concentrations of BN in brain and peritoneal fat were below detectable limits (< 0.1 μg/g tissue). Urine and fecal excretion of BN accounted for < 2% of the dose after 24 h. In vitro metabolism studies using a postmitochondrial microsome fraction of liver, lung, and kidney suggest that bruceantin is inactivated by an NADPH-dependent enzyme present in liver but not in lung or kidney.*

* Supported by contract NO1-CM-87162 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare

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Introduction

Bruceantin (NSC 165563; Fig. 1) is a member of the quassinoid or simaroubolide group of compounds and was originally isolated from the stem bark of *Brucea antidysenterica* (J. F. Mill) (Kupchan et al., 1973, 1975). The drug has shown good activity against KB cells in vitro as well as against B16 melanoma, L1210 leukemia, and P388 leukemia in the mouse (Hartwell, 1976; Douros and Suffness, 1978). Bruceantin does not appear to have antibacterial activity and shows only weak activity (10–1000 μg/ml) against some strains of yeast (Suling, unpublished results). Studies of the mechanism of action of bruceantin suggest that the compound primarily inhibits initiation of protein synthesis in susceptible cells (Liao et al., 1976).

Preclinical toxicological studies of bruceantin in mice, dogs, and monkeys have been completed (Castles et al., 1976) and the drug is now in clinical trials.

The current study involved the disposition and excretion of bruceantin in both normal and solid L1210 tumor-bearing mice as well as the in vitro metabolism of the drug. Both normal and tumor-bearing mice were studied to compare levels of drug in the tumor relative to non-malignant tissues, and to determine possible effects of the malignancy on drug disposition. A sensitive and specific microbiological assay was developed and used to quantify levels of bruceantin in urine and tissues.

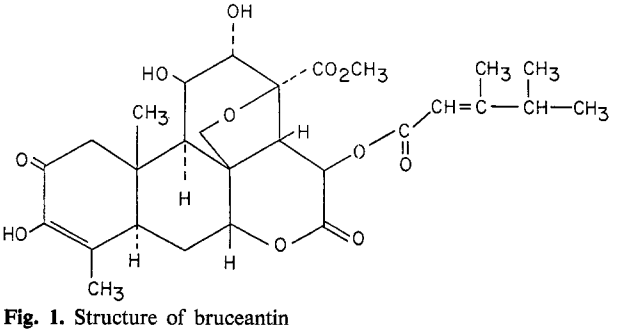

Fig. 1. Structure of bruceantin

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Materials and Methods

Chemicals. Bruceantin was obtained from the National Cancer Institute, Bethesda, Maryland. Thin-layer chromatography of the drug preparation, with three solvent systems, yielded a single migrating component after charring with 50% sulfuric acid. Glucose-6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from the Sigma Chemical Co., St. Louis, Missouri.

Experimental Protocol. Male BDFl mice (20 g) were used throughout this study. Tumor-bearing mice were obtained by subcutaneous implantation of 10^6 L1210 ascites cells per mouse. Animals were held for seven days at which time the average tumor weight was 307 mg and advanced generalized disease was evident.

Normal and tumor-bearing mice were given single iv doses of bruceantin (1.5 mg/kg; approx. LD_{10}) via the tail vein. The delivery vehicle was 3% ethanol and 0.05% Tween 80 in 0.9% saline. Control mice were injected with the vehicle alone. At various time intervals up to and including 60 min, three groups of 5 or 10 mice were anesthetized and blood was taken by cardiac puncture with heparinized syringes. The blood was pooled into tubes held in ice. Two groups of 10 mice were used for later time intervals. Tissues were excised, blotted on saline-saturated gauze pads, weighed, and homogenized in nine parts of cold 0.9% saline. Urine and feces were collected over a period of 24 h from mice that were housed in metabolism cages. The urine was collected into chilled tubes and all samples were stored at −20° until they were extracted and assayed. Fecal material was homogenized in nine parts of saline prior to extraction.

Samples were extracted three times with equal volumes of chloroform. Emulsions were centrifuged in order to hasten the separation of layers. The lower chloroform layers from the three extractions were pooled, concentrated to about 0.5 ml using a Buchler Evapomix evaporator, quantitatively transferred to one dram glass vials, and evaporated to dryness under a stream of nitrogen. The dried extracts were dissolved in 0.1 to 0.4 ml of chloroform and assayed. All chloroform extracts were stored at −20° in vials which were sealed with screwcaps with Teflon liners.

Blood clearance curves and half-lives for bruceantin were obtained by fitting a poly-exponential equation to the data using a nonlinear least squares program (Metzler et al., 1974). Initial parameter estimates were obtained from exponential curve stripping (Sedman and Wagner, 1976).

Assay. Bruceantin levels were determined with a microbial assay of the agar disc diffusion type using a mutant strain of yeast which had been tentatively identified as *Candida macedoniensis*. The mutant strain, designated as *C. macedoniensis* SRI 1021 (CM1021), was isolated by exposure of the cells to ultraviolet light followed by replica plating of colonies, which were derived from the cell survivors, onto solid medium with and without bruceantin (0.01 to 0.1 μg/ml). Replicates that failed to grow on the drug supplemented medium, but grew on the unsupplemented medium, were picked and examined for susceptibility to bruceantin. CM1021 was found to be 20-fold more sensitive to bruceantin than the original parent strain.

The medium used for both the subculture of CM1021 and assay of bruceantin contained 0.1% dipotassium hydrogen phosphate, 0.3% sodium nitrate, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.001% ferrous sulfate, 0.025% Bacto yeast extract (Difco Laboratories, Detroit, Michigan) and 0.3% sucrose. The medium used for assay was supplemented with 0.75% Difco agar. Assay plates were prepared as follows: an overnight subculture of CM1021 (37° incubation for 18–24 h) was centrifuged and the cells were washed twice with 0.9% saline and resuspended in saline to about 2×10^7 viable cells/ml. Ten milliliters of the standardized cell suspension were mixed with each liter of melted agar medium, dispensed into 15 × 100-mm disposable plastic Petri dishes (6 ml/assay plate), and allowed to solidify at room temperature. Assay plates were stored at 5° for up to one month.

A stock solution of bruceantin (3 mg/ml in dimethylsulfoxide) was stored at −20° and used for the preparation of standard curves. This solution was stable for at least six months as determined by the simultaneous assay of a reference solution (2 μg/ml), which was prepared fresh at the time of each assay. Standard curves were prepared by serial twofold dilutions of the reference solution in ice-cold chloroform. Twenty microliters of each dilution were applied to filter paper discs (Schleicher and Schuell Co., Keene, NH; 0.63 cm diameter), the chloroform was allowed to evaporate, and the discs were placed onto the surfaces of the assay plates. Each assay plate contained three standard concentrations (1.0, 2.0, and 4.0 or 2.5, 5.0, and 10 μg/ml) in addition to either two dilutions of a test sample or two different test samples. All samples were assayed in triplicate. The plates were incubated at 37° for 18 to 24 h at which time zones of growth inhibition were measured to the nearest 0.1 mm with a vernier dial caliper. Standard curves were calculated according to the method of least squares using the relationship between the logarithm of the drug concentration and the zone diameters. Inhibition zone diameters from the test samples were converted to μg of bruceantin using the above standard curves.

Microsomal Enzyme Preparation. Liver, kidney, and lung were obtained from anesthetized male BDFl mice (20–25 gm) that were first bled by cardiac puncture. The 9000 *g* supernatant (S-9) of homogenates of the above organs was prepared as described by others (Ames et al., 1975).

Microsomal Enzyme Assay Conditions. The assay mix contained (per ml) 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADP, 100 μmol phosphate buffer (pH 7.4), 10 μg bruceantin, and S-9 (0.1–0.3 ml). All components, except the S-9 fraction, were preincubated at 37° for 10 min. The reaction was initiated by the addition of S-9 and incubation was continued for various time periods. Reactions were stopped by the addition of 2 ml of chloroform to 0.5 or 1 ml of mix. Each mixture was vortexed, centrifuged, and the chloroform layer transferred to a two-dram vial. A second chloroform extraction was done on the aqueous phase and the two extracts were pooled and evaporated to dryness under a stream of nitrogen. The dried extracts were then dissolved in 0.5 or 1 ml of chloroform and assayed for bruceantin.

Bioautography. Thin-layer chromatography of the chloroform extracts of homogenized tissues, urine, and feces was done on glass fiber sheets impregnated with polysilicic acid gel (Gelman type-SA) using chloroform/acetone (C/A; 9 : 1), chloroform/methanol (C/M; 95 : 5), benzene/methanol (B/M; 95 : 5), and hexane/ether (H/E; 4 : 1) solvent systems. After development, the chromatograms were air-dried and placed onto the surface of agar assay medium that had been seeded with CM1021 and solidified (1.5% agar) in Pyrex baking dishes. After 60 min, the chromatograms were removed from the assay plates and the plates were incubated at 37° for 16 to 18 h. The position of bruceantin on the chromatograms was visualized as a distinct zone of growth inhibition on the assay plates. Also, chloroform solutions of bruceantin and chloroform extracts from the microsomal enzyme reaction studies were chromatographed as described and the components visualized by spraying with 50% sulfuric acid followed by charring at 130°. Chromatograms were also sprayed with 5% ferric chloride in 95% ethanol (Kupchan et al., 1975) for the detection of bruceantin and possible metabolites.

Table 1. Tissue distribution of bruceantin (1.5 mg/kg) in normal (N) and L1210-bearing (T) BDF1 mice[a]

Sample[b]		Bruceantin μg/gm ± SE at					
		5 min	15 min	30 min	60 min	120 min	240 min
Kidney	(N)	0.45 ± 0.040	0.47 ± 0.12	0.25 ± 0.026	0.27 ± 0.028	ND[c]	0.17 ± 0.010
	(T)	0.81 ± 0.088	0.42 ± 0.049	0.29 ± 0.041	0.28 ± 0.020	0.23 ± 0.010	0.17 ± 0.0
Liver	(N)	0.36 ± 0.027	0.25 ± 0.023	0.20 ± 0.003	0.24 ± 0.013	ND	0.16 ± 0.005
	(T)	0.39 ± 0.040	0.38 ± 0.016	0.34 ± 0.068	0.29 ± 0.029	0.25	0.15 ± 0.005
Spleen	(N)	1.3 ± 0.50	0.96 ± 0.47	1.2 ± 0.23	0.83 ± 0.024	ND	0.29 ± 0.044
	(T)	0.63 ± 0.13	1.1 ± 0.31	0.96 ± 0.36	0.58 ± 0.15	0.24 ± 0.020	0.24 ± 0.005
Pancreas	(N)	0.82 ± 0.12	1.0 ± 0.12	0.82 ± 0.21	0.94 ± 0.11	ND	0.49 ± 0.020
	(T)	1.3 ± 0.35	1.9 ± 0.22	0.94 ± 0.26	1.2 ± 0.26	0.90 ± 0.010	0.65 ± 0.044
Sm. Int.	(N)	0.69 ± 0.13	0.74 ± 0.15	0.53 ± 0.032	0.73 ± 0.078	ND	0.26 ± 0.005
	(T)	0.81 ± 0.24	0.95 ± 0.14	0.67 ± 0.089	0.60 ± 0.055	0.56 ± 0.079	0.32 ± 0.030
Lg. Int.	(N)	0.92 ± 0.28	1.0 ± 0.35	0.33 ± 0.024	0.64 ± 0.029	ND	0.16 ± 0.020
	(T)	0.76 ± 0.24	0.74 ± 0.21	0.69 ± 0.15	0.58 ± 0.10	0.59 ± 0.074	0.26 ± 0.065
Lung	(N)	5.5 ± 2.7	ND	3.8 ± 1.2	1.8 ± 0.18	ND	0.10 ± 0.0
	(T)	11.0 ± 2.5	6.3 ± 2.2	2.7 ± 0.37	2.5 ± 1.0	0.97 ± 0.12	0.36 ± 0.050
Brain	(N)	< 0.1	ND	ND	ND	ND	ND
	(T)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Fat	(N)	ND	ND	ND	ND	ND	ND
	(T)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Tumor		0.27 ± 0.030	0.25 ± 0.040	0.21 ± 0.032	0.29 ± 0.027	0.22 ± 0.0	0.18 ± 0.001

[a] Assays were done as described in Materials and Methods
[b] Samples were pooled from 5 or 10 mice per group. Data is from 3 groups (5 to 60 min) and 2 groups (120 and 240 min)
[c] ND = not determined

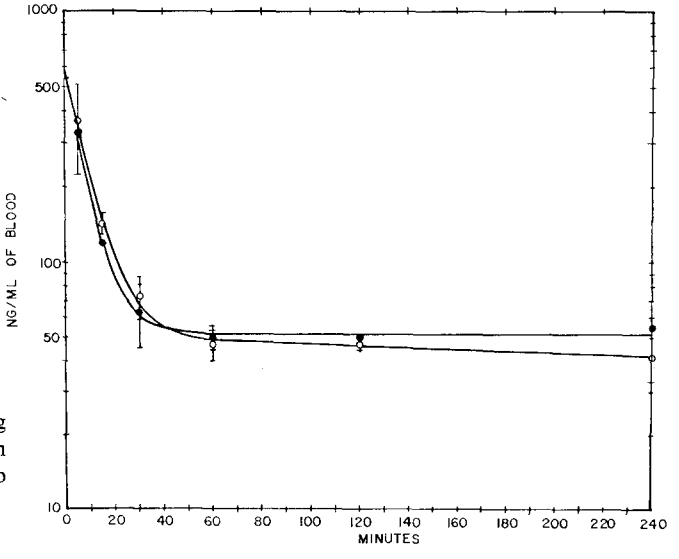

Fig. 2. Blood levels of bruceantin in normal (○) and tumor-bearing (●) BDF1 mice following a single IV injection of 1.5 mg/kg. Each point represents the mean value for pooled blood from 3 groups (5 to 60 min) or 2 groups (120 and 240 min) of mice ± SE

Results

Assay and Drug Recovery. The use of a three-point standard curve and 4-fold range of concentrations gave good linearity when the relationship between the diameter of the zone of inhibition and the logarithm of the drug concentration was applied. Accuracy of the assay was determined with nine replications of individually weighed samples of bruceantin which were assayed on different days. The coefficient of variation (SD/$\bar{X}$) was calculated to be 0.09.

The recovery and stability of bruceantin during the extraction and assay were determined by the addition of a known amount of drug to appropriate tissue homoge-

nates, blood, urine, and feces. Triplicate determinations on each type of sample yielded recoveries that ranged from 91 to 121% with an average coefficient of variation of < 10%. There was no indication that chloroform extractable material from the biologic samples interfered with the assay.

Specificity of the assay method for bruceantin was evaluated by bioautography as described. This method allowed detection of < 0.05 μg of bruceantin, the lowest amount spotted. Except for blood and lung, chloroform extracts of tissues which were excised up to and including 60 min after dosing and which had biologic activity were bioautographed. Chloroform extracts of 0–24 h urine and feces were also examined. Chromatograms were developed in three solvent systems. R_f values for a standard solution of bruceantin in chloroform were as follows: C/A, 0.35; B/M, 0.55; C/M, 0.79. A single biologically active component with an R_f similar to that of bruceantin was observed with all of the samples that were examined. Movement of the biologically active component, however, was hindered during chromatography of extracts of liver, kidney, and pancreas from treated mice. When these same extracts were supplemented with a known amount of bruceantin, a single biologically active component was still observed.

Animal Studies. The distribution of bruceantin in tissues of both normal and tumor-bearing mice is presented in Table 1. Within 5 min of dosing, bruceantin localized in all tissues examined except brain and peritoneal fat. Highest levels of the drug were found in the lung, pancreas, intestine, and spleen. Lower drug levels occurred in the liver, kidney, and tumor. Except for lung, tissue levels of the drug at 4 h were 20 to 60% of those at 5 min. Levels of bruceantin in the tissues of normal and tumor-bearing mice were similar.

Blood clearance curves for bruceantin are presented in Figure 2. Decay of drug with time was biphasic with little difference observed between normal and tumor-bearing mice. A rapid distribution or α phase, with half-lives of 6 and 5 min for normal and tumor-bearing mice, respectively, was observed during the first 40 min after IV dosing. This was followed by a much less rapid β phase. Accurate half-lives for β phase elimination could not be obtained because of the limited observation period, but the estimated half-lives were probably > 0.5 day. Average zero time intercepts for the α and β phases were 550 and 51 ng/ml respectively.

For normal mice, elimination of bruceantin in urine and feces over a 24 h period accounted for only 0.72% and 0.68% of the dose, respectively. Tumor-bearing mice excreted similar amounts of the drug. In order to determine if bruceantin was excreted in bile, gall bladders from treated normal mice were excised at different times, placed upon filter paper discs, ruptured with a needle, and assayed for activity in terms of bruceantin equivalents. Estimated concentrations in bile between 5 and 60 min after dosing were within the range of 1.5 to 13 μg of bruceantin equivalents per ml of bile.

Table 2. Effect of mouse S-9 microsome fractions of lung, kidney, and liver on bruceantin activity[a]

Tissue	ML S-9 added	NADP	Bruceantin	
			μg recovered[b]	Relative recovery[c]
Lung	0.3	–	8.59	
	0.3	+	8.18	0.96
Kidney	0.1	–	10.2	
	0.1	+	9.91	0.97
	0.3	–	9.93	
	0.3	+	9.66	0.97
Liver	0.1	–	9.19	
	0.1	+	1.07	0.2

[a] Each reaction mix contained (per ml) bruceantin (10 μg, 0.02 μmol), $MgCl_2$ (8 μmol), KCL (33 μmol), G-6-P (5 μmol), phosphate (100 μmol), S-9 fraction, and, when added, NADP (4 μmol). Incubation was for 40 min at 37°

[b] μg of bruceantin recoverable into $CHCl_3$ after 40 min incubation

[c] Ratio of μg bruceantin recovered in the presence of NADP to μg recovered in the absence of NADP

In vitro Metabolism Studies. Neither lung nor kidney microsomes appeared able to inactivate bruceantin as evidenced by almost complete recovery of the drug from the reaction mixes after 40 min of incubation (Table 2). In contrast to these results, only 20% of the bruceantin added to the reaction mixture containing mouse liver microsomes was recoverable after incubation. Metabolism of bruceantin by liver microsomes took place only in the presence of the NADPH generating system.

The time course of inactivation of bruceantin by mouse liver microsomes is presented in Figure 3. Fifty percent of the bruceantin in the reaction mix was converted to an inactive product(s) in 4.3 min.

The nature of the product(s) formed from bruceantin in the presence of mouse liver microsomes was examined by TLC. Microsomes were incubated with bruceantin for 40 min in the presence and absence of NADP and also in the absence of bruceantin but with NADP. Chloroform extracts of the three preparations were chromatographed in the C/A solvent system, air-dried, and again developed in the H/E solvent system in order to move nonpolar lipid components closer to the solvent front. A component with an R_f value identical to that of bruceantin ($R_f = 0.5$) was detected only from the preparation not containing NADP. The chloroform extract of the preparation containing both bruceantin and NADP

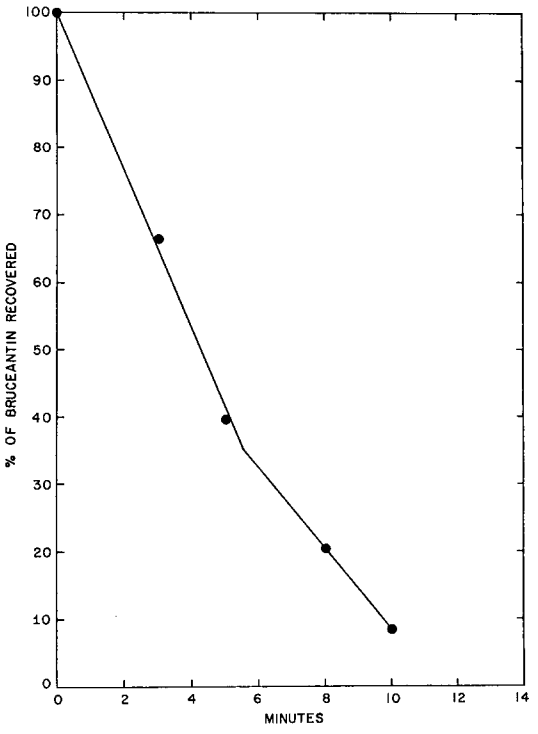

Fig. 3. Rate of inactivation of bruceantin by mouse liver S-9 microsome fraction. Bruceantin (0.02 μmol/ml) was incubated for 10 min at 37° in a mixture of (per ml) $MgCl_2$ (8 μmol), KCl (33 μmol), G-6-P (5 μmol), phosphate (100 μmol), and NADP (4 μmol). The reaction was initiated by the addition of 0.3 ml of mouse liver S-9 fraction per ml of mixture. Samples were taken at various times and immediately added to tubes containing chloroform. Chloroform extracts were assayed for bruceantin and the % of bruceantin recovered was calculated. Each point represents the average percentage recovery from duplicate samples.

yielded a new component with an R_f of 0.1. This component was not present in the chloroform extracts of the other two mixes. When the chromatograms were treated with ferric chloride, both bruceantin and the potential metabolite (R_f = 0.1) yielded a distinctive gray color.

Discussion

Bruceantin is a novel natural product that shows good activity in both leukemias and solid tumors in mice and is potentially an agent with a broad antitumor spectrum (Douros and Suffness, 1978). Although the drug has been entered into Phase I clinical trials, there have been no reports in the literature that deal with its pharmacology in laboratory animals or humans.

The assay described here should be applicable to pharmacologic studies of bruceantin in humans. The drug can be extracted from biologic samples into chloroform and assayed without further clean-up procedures. The assay is therefore easy to perform, inexpensive, and appears to be specific. Although other drugs might be administered in combination with bruceantin, some of which might interfere with the assay, many drugs may not be extractable into chloroform. Antibacterial chemotherapeutic agents should not interfere with the assay since the indicator strain is a yeast.

Following an IV dose, bruceantin was observed to localize rapidly in all excised tissues except for brain and fat where drug levels were below detectable limits (Table 1). Except for lung, the slow rate of elimination of drug from tissues correlated with the extended β-phase half-life of the drug in the blood. The reported apparent cumulative toxicity of bruceantin in dogs and monkeys following 10 daily IV doses (Castles et al., 1976) might be explained by these results.

Levels of bruceantin in lung were found to be 10-fold or more higher than other tissues at the earlier time points. These levels decreased to about 3% of levels at 5 min. The initial high levels of drug in lung may have been due to an incomplete solubilization of drug in the injection vehicle or rapid uptake during first passage through the tissue. With the former, suspended particles of drug could lodge in the microcapillaries of the lung where they would eventually dissolve. The results of a preliminary study in which bruceantin was administered IP indicated that, under these conditions, levels of bruceantin in the lung were no greater than those in spleen, pancreas, or intestine.

No major differences were found between the disposition of bruceantin in normal and tumor-bearing mice. It was demonstrated, however, that the drug localized in the tumor where concentrations were comparable to those in liver and kidney.

Bruceantin was excreted in both urine and feces over a 24-h period. Recovery, however, accounted for < 2.0% of the dose. Levels of drug remaining in the animals could not account for the difference, suggesting that the drug underwent metabolism prior to excretion. In vitro studies demonstrated that bruceantin was metabolized in the presence of the postmitochondrial fraction of mouse liver but not kidney or lung. The requirement for NADPH in the reaction mixture suggests that microsomal mixed function oxidases are involved. Further, the chloroform-extractable metabolite(s) was not active against the yeast that was used for the assay of bruceantin. One might infer that the metabolism of bruceantin by the liver is therefore a detoxification or inactivation process.

TLC of chloroform extracts of liver microsomal reaction mixtures containing bruceantin indicated the formation of a relatively more polar component than bruceantin after incubation. The identity of this potential metabolite is not yet known. Studies are now in progress to identify this and other possible metabolites of bruceantin.

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Received March 15/Accepted May 21, 1979