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ETHACRYNIC ACID—AN INHIBITOR OF L-ASPARAGINE SYNTHETASE

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Abstract—Ethacrynic acid, a clinically useful diuretic, has been shown to inhibit L-asparagine synthetase from leukemia 5178Y resistant to L-asparaginase (L5178Y/AR) *in vitro*. This inhibition is thought to involve the formation of an adduct between ethacrynic acid and sulfhydryl functions on the enzyme; the adduct is not readily reversible even when another thiol, such as dithiothreitol, is used to displace the acid. A series of analogs of ethacrynic acid were examined as inhibitors of L-asparagine synthetase. Only one proved to be superior to the title compound; it was 2-(1-carboxymethoxy-4-chloro-2-naphthyl)3,6-diethyl-6-(1-carboxymethoxy-4-chloro-2-naphthoyl)-5,6-dihydro-[4H]-pyran (Compound D). There was no correlation between the diuretic or natriuretic potency of the series of compounds examined and their ability to inhibit L-asparagine synthetase activity. There was also no correlation between the ability of these agents to inhibit L-asparaginase from *Dasyprocta agouti* (another sulfhydryl enzyme) and L-asparagine synthetase from L5178Y/AR. In every case, ethacrynic acid and its analogs interrupted the utilization of ammonia by L-asparagine synthetase to a greater degree than the utilization of L-glutamine. *In vivo*, the inhibition of L-asparagine synthetase from L5178Y/AR by ethacrynic acid was feeble, while the analogous enzyme from pancreas was inhibited to a significant degree.

Ethacrynic acid ([2,3-dichloro-4-(2-methylenebutyryl)-phenoxy]acetic acid) is a potent and clinically useful diuretic [1]. In addition, the drug is known to interrupt a considerable number of cellular processes: sodium fluxes in muscle [2]; ion fluxes in bladder [3]; mitochondrial metabolism [4]; glycolysis [5]; and the inflammatory process [6]. In many cases, the action of ethacrynic acid has been ascribed to, or associated with the blockade of critical sulfhydryl functions on the enzyme or organelle under study. Chemically, this blockade is most likely achieved via covalent reaction at the aryloxyacetic moiety (Fig. 1).

Since we are engaged in an extensive search for inhibitors of tumoral L-asparagine synthetase, and inasmuch as evidence in the literature indicates that sulfhydryl groups are involved in the catalytic activity of the enzyme, we were prompted to examine ethacrynic acid as a candidate inhibitor. The present report summarizes the inhibitory behavior of the drug *in vitro* and *in vivo*.

MATERIALS AND METHODS

Supplies

Ethacrynic acid and its analogs were provided by Merck, Sharp & Dohme, West Point, Pa. L-Asparagine was purchased from Schwartz Mann, Rockville, Md., and L-[4-^{14}C]-aspartic acid from the Biochemical Nuclear Co., Burbank, Calif. All other chemicals were of the highest quality obtainable.

Enzymes

Glutamate oxaloacetate transaminase (GOT) (EC 2.5.1.1, sp. act. 180 I.U./mg of protein) and malate dehydrogenase (MDH) (EC 1.1.1.37, sp. act. 1100 I.U./mg of protein) were purchased from Boehringer, New York, N.Y., as ammonium sulfate suspensions.

L-Asparaginase (*EC* 3.5.1.1)

From agouti serum. Serum from the agouti, *Dasyprocta agouti*, was prepared from blood obtained by cardiac puncture. A preliminary purification of L-asparaginase was carried out by adding solid ammonium sulfate to 30 per cent saturation; the scant precipitate which formed was discarded. Additional solid ammonium sulfate was added, to 50 per cent saturation; the precipitate was collected, redistributed in 0·1 M Tris-HCl buffer, pH 7·5, containing 10% glycerol and 0·001 M dithiothreitol, dialyzed against several changes of the same buffer and stored at −20°; this was used as the source of L-asparaginase from agouti serum (sp. act. 20 I.U./mg of protein).

From E. Coli. L-Asparaginase from *Escherichia coli* (sp. act. 340 I.U./mg of protein) was a donation to the National Cancer Institute from the Merck Institute for Therapeutic Research, Rahway, N.J.

L-Asparagine synthetase [*EC* 6.3.5.4, *L-aspartate: L-glutamine amido ligase* (*AMP*)]

From leukemia 5178Y made resistant to L-asparaginase (*L5178Y/AR*). Male BDF$_1$ mice weighing

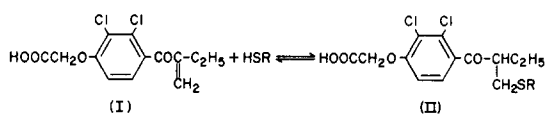

Fig. 1. Ethacrynic acid (I) reacts with sulfhydryl groups (HSR) to form an ethacrynic acid–sulfhydryl complex (II). This complex (II) can further undergo a sulfide–sulfhydryl exchange in the presence of sulfhydryl-containing proteins.

between 18 and 20 g, were injected by the subcutaneous route with a suspension of 10^6 cells of leukemia 5178Y made resistant to L-asparaginase (L5178Y/AR) by repeated subcurative doses of the enzyme. Seven to ten days later, tumors were collected and homogenized (1:10, w/v) in 0·1 M Tris-HCl buffer, pH 7·6, containing 0·5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged in the cold at 100,000 *g* in a Beckman L2-65B ultracentrifuge for 30 min and the supernatant (sp. act. 0·001 I.U./mg of protein) was collected and used as the source of L-asparagine synthetase from tumor. For the kinetic analysis of the type of inhibition produced by ethacrynic acid, the enzyme was partially purified (6-fold) by heating at 50° for 10 min after treating with ATP, $MgCl_2$, L-glutamine and ammonium chloride each at a concentration of 0·01 M. The heated extract was immediately chilled on ice and centrifuged at 20,000 *g* for 30 min to remove the precipitated proteins and nucleic acids. The supernatant was brought to 30 per cent saturation with solid ammonium sulfate and then centrifuged at 18,000 *g*. The precipitate was discarded and the supernatant brought to 50 per cent saturation with solid ammonium sulfate. The resulting precipitate was suspended in 5 mM Tris-HCl buffer, pH 7·6, containing 0·5 mM EDTA, 1 mM dithiothreitol and 10% glycerol, and dialyzed against three changes of the same buffer for 3 hr.

From mouse pancreas. Pancreata from twenty normal BDF_1 mice were collected at 4° and homogenized (1:10, w/v) in 0·1 M Tris-HCl buffer, pH 7·6, containing 1 mM dithiothreitol and 0·5 mM EDTA. The homogenate was centrifuged in the cold for 3 min at 18,000 *g* and the supernatant (sp. act. 0·001 I.U./mg of protein) was used as the source of L-asparagine synthetase from mouse pancreas.

Protein was determined according to the method of Lowry *et al.* [8].

Measurement of L-asparaginase

Measurement of L-asparaginase activity was carried out in two stages: incubation and assay. Incubations of the agouti and *E. coli* enzymes with 0·05 M L-asparagine in 0·1 M Tris-HCl buffer, pH 8·4, in the presence or absence of one of the putative inhibitors, were conducted at 37° for 30 min. The reaction was terminated with one-tenth volume of 2 N HCl followed in 10 min by one-tenth volume of 2 N NaOH. L-Aspartic acid was then measured in an aliquot of the incubation mixture by an enzymatic spectrophotometric technique [9]. In no case was more than 10 per cent of the substrate hydrolyzed; additionally, aliquots for the assay were sufficiently small so that no inhibition of the reagent enzymes (GOT and MDH) by the drugs was observed.

Measurement of L-asparagine synthetase

L-Asparagine synthetase was measured by an enzymatic radiometric technique, essentially as described earlier [10], with the following modifications. In a final volume of 10 µl were admixed: 0·1 µmole ATP and magnesium chloride, 0·2 µmole L-glutamine or 0·5 µmole ammonium chloride, 0·125 µCi (0·0056 µmole) L-[4-^{14}C]-aspartic acid (sp. act. 22·2 µCi/µmole) and 5 µl crude or partially purified L-asparagine synthetase from tumor or pancreas; this mixture was incubated for 30 min at 37°. After incubation the vessels were heated at 95° for 5 min and any residual unreacted L-[4-^{14}C]-aspartic acid was then β-decarboxylated by addition to each vessel of 50 µl of a decarboxylation reagent consisting of 6·8 mM α-ketoglutaric acid, and 8·5 mM zinc sulfate, in 0·66 M sodium acetate buffer, pH 5·0, containing 0·2 mg/ml of GOT. The open vessels were incubated at 37° for 4 hr, whereafter 2 I.U. of L-asparaginase from *E. coli* was added. At the same time, a droplet (5 µl) of a saturated solution of barium hydroxide was deposited on the underside of each vessel's lid to trap [^{14}C]-O_2 arising from the zinc-catalyzed β-decarboxylation of oxaloacetic acid, arising in turn from the transamination with α-ketoglutaric acid of any L-[4-^{14}C]-aspartic acid produced by the amidohydrolysis of L-[4-^{14}C]-asparagine. When inhibitor constants were being investigated, sufficient drug was lyophilized on the bottom of the plastic reaction vessel so that, on reconstitution with the 10 µl final reaction volume, the intended molarities were achieved. This procedure led to no measurable alteration of the absorption spectrum of the inhibitor or of the potency of inhibition. Upon reconstitution, the lyophilized drug dissolved instantaneously.

Antitumor studies

Seven days after intraperitoneal transplantation into BDF_1 mice of 1×10^5 cells of L5178Y/AR, treatment was started with clinical formulations of ethacrynic acid or compound D at the doses indicated. Control subjects received saline.

Attempts to prevent or reverse inhibition of L-asparagine synthetase by ethacrynic acid

Aliquots of the 105,000 *g* supernatant of a 1:10 (w/v) homogenate of L5178Y/AR in 0·1 M Tris-HCl buffer, pH 7·6 were rendered 0·01 M in L-glutamine, 0·1 M in NH_4Cl, 0·001 M in L-aspartic acid, 0·01 M in ATP $MgCl_2$, or 0·001 M in dithiothreitol either immediately before (= prevention) or 30 min after (= reversal) the addition of ethacrynic acid to a final concentration of 1×10^{-3} M. Ten min later, aliquots of these incubation mixtures were diluted (1:3, v/v) with water, and assayed radiometrically for L-asparagine synthetase activity.

Kinetic analyses

For the double reciprocal plots, extracts of L5178Y/AR (sp. act. 0·0058 I.U./mg of protein) were incubated for 30 min in the presence of 0, 0·5 or 1·0 mM ethacrynic acid, and different concentrations of L-glutamine (2·5, 5·0, 10·0 or 20·0 mM), ammonium chloride (6·25, 12·5, 25·0 or 50·0 mM) or L-aspartate (0·5, 1·0, 2·0 or 4·0 mM) and 0·1 mM ATP and $MgCl_2$. The production of L-[4-^{14}C]-asparagine was quantitated as described earlier.

RESULTS

When ethacrynic acid is incubated at a concentration of 1×10^{-3} M with a crude (105,000 *g* supernatant) preparation of tumoral L-asparagine synthetase, *in vitro*, the enzyme is inhibited strongly

Table 1. Inhibition *in vitro* of L-asparagine synthetase and L-asparaginase by ethacrynic acid and its analogs*

Compound	Mean % inhibition of L-asparagine synthetase		Mean % inhibition of L-asparaginase		Natriuretic activity in dogs
	Amide-donors L-Glutamine	Ammonia	*E. coli*	Agouti	
A. [2,3-Dichloro-4-[2-(mesylmethyl)butyryl]phenoxy]acetic acid	30	46	0	0	5
B. 2,3'-Dichloro-4'-(2-diethylaminoethoxy)-2-methylenebutyrophenone *p*-toluenesulfonate	34	71	0	29	±
C. N-[2,3-dichloro-4-(2-methylenebutyryl)phenoxy acetyl]-L-alanine	7	32	0	25	±
D. 2-(1-Carboxymethoxy-4-chloro-2-naphthyl)-3,6-diethyl-6-(1-carboxymethoxy-4-chloro-2-naphthoyl)-5,6-dihydro-[4H]-pyran	81	90	0	18	0
E. N-(carboxymethyl)-[2,3-dichloro-4-(2-methylenebutyryl)-phenoxy]acetamide	22	60	0	35	4
F. 4-(α-Ethylacrylyl)-1,3-phenylenebisoxy-acetic acid	14	29	1	21	0
G. [2-Methyl-3-chloro-4-(2-ethylidenebutyryl)phenoxy]acetic acid	9	26	0	53	6
H. [2,3-Dichloro-4-(2-bromoisovaleryl)phenoxy]acetic acid	12	15	0	29	5
I. [2,3,5-Trimethyl-4-(2-methylenebutyryl)phenoxy]acetic acid	4	13	0	27	±
J. 2-(2,3-Dichloro-4-carboxymethoxyphenyl)-3,6-diethyl-6-(2,3-dichloro-4-carboxymethoxybenzoyl)-5,6-dihydro-[4H]-pyran	16	55	0	23	0
K. [3-Chloro-4-(2-methylenebutyryl)phenoxy]acetic acid	37	54	4	23	4
L. [4-(2-Methylenebutyryl)-1-naphthyloxy]acetic acid	32	48	2	40	5
M. [2-Isopropyl-4-(2-methylenebutyryl)-5-methylphenoxy]acetic acid	13	35	8	27	0
N. [2,3-Dichloro-4-(2-methylbutyryl)phenoxy]acetic acid	7	22	0	20	±
O. [2,3-Dichloro-4-(2-chloroisovaleryl)phenoxy]acetic acid	23	24	16	36	±
P. [2,5-Dichloro-4-(2-methylenebutyryl)phenoxy]acetic acid	31	44	0	27	1
Q. [2,3-Dimethyl-4-(2-methylenebutyryl)phenoxy]acetic acid	11	16	0	23	5
R. [2,3-Dichloro-4-(2-ethylidenebutyryl)phenoxy]acetic acid	11	17	0	28	5
S. Ethacrynic acid	58	70	0	21	6
Ethacrynic acid (2.5×10^{-3} M)	76	84			

* Inhibition of L-asparagine synthetase from L5178Y/AR was measured essentially as described in Methods, with the exception that the reaction volume was increased to 45 μl and that the ethacrynic acid derivatives listed in the Table were added to the incubation mixture to a final concentration of 1×10^{-3} M. Vessels receiving 10 μl of H_2O instead of drug served as standard. Both L-glutamine and NH_3 (in the form of NH_4Cl) were used as amido donors. The values represent the mean of four experiments.

Inhibition of L-asparaginase was measured spectrophotometrically using L-asparaginase from *E. coli* and agouti serum in the presence or absence of drugs listed in the Table at a final concentration of 1×10^{-3} M.

In the assay of natriuresis, female animals are starved overnight, anesthetized with phenobarbital, creatinine-primed, catheterized and infused with phosphate buffer at a rate of 3 ml/min. The drug was then given i.v. at 5 mg/kg over a period of 5 min, and 15-min collections of urine were taken over a period of 2 hr. The data recorded are the average of the two highest consecutive 15-min collections. The natriuretic response is scored from 0 to 6 according to the criteria shown below:

Score	Dog assay (5 mg/kg, i.v., stat dose) (μ-equiv. Na^+ excreted/min)
±	Active only above 5 mg/kg
0	0–99
1	100–300
2	301–500
3	501–700
4	701–900
5	901–1200
6	Above 1200

(Table 1). Many of the congeners of ethacrynic acid also share this property. In every case, the inhibition of the synthesis of L-asparagine is greater when ammonia is the substrate than when L-glutamine is the amide donor. This feature is illustrated graphically in Fig. 2. It is of interest that the most potent inhibitor detected in the present series (Table 1), 2-(1-carboxymethoxy-4-chloro-2-naphthyl)-3,6-diethyl-6-(1-carboxymethoxy-4-chloro-2-naphthoyl)-5,6-dihydro-[4H] pyran (Compound D), is, on the face of it, structurally dissimilar to ethacrynic acid, lacking as it does the vicinyl chlorine atoms and the reactive 2-methylene-butyryl substituent, a functionality known to form sulfhydryl adducts according to the scheme presented in Fig. 1. The basis for the high reactivity of compound D is, then, unclear.

Also, apparent from the data presented in Table 1 is the conclusion that neither the parent compound, ethacrynic acid, nor its analogs are potent inhibitors of L-asparaginase from *E. coli*. Although one intersulfide bridge does stabilize the molecule [11], this oncolytic hydrolase is known to be exceedingly resistant to inhibition by sulfhydryl reagents [12]. On the other hand, L-asparaginase from the serum of agouti, *Dasyprocta agouti*, is sensitive to inhibition by ethacrynic acid and certain of its analogs; thus, this hydrolase is similar in behavior to other mammalian L-asparaginases which have been demonstrated to be similarly susceptible to sulfhydryl blockade [12].

It should be pointed out that the correlation between inhibition of L-asparagine synthetase and agouti L-asparaginase is imperfect. Thus, compound

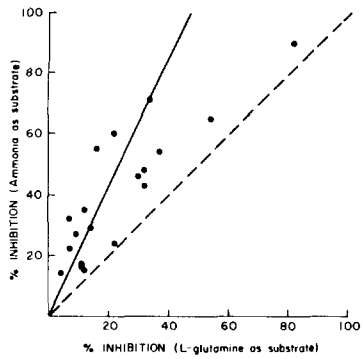

Fig. 2. Correlation between inhibition by Compounds A S (●) of Table 1 of L-asparagine synthetase activity from L5178Y/AR, using L-glutamine and ammonia as substrates. Individual pairs of observations are shown, as well as the line representing the least squares best fit to the data (solid line). Also shown is the diagonal (broken line) which would represent the data if inhibition were equal using the two substrates.

D (Table 1) inhibits L-asparagine synthetase powerfully but agouti L-asparaginase weakly. Disparities such as these may be a result of steric factors affecting the entry of the reagent into the sulfhydryl site on the respective enzyme. Also documented in Table 1 is the failure of the natriuretic potency of this family of compounds to correlate with their enzyme-inhibitory activity. This descrepancy is most prominent in the case of compound D, which powerfully inhibited L-asparagine synthetase, but produced no natriuresis in creatinine-primed dogs. It is likely that differential susceptibility to adduct formation of protein-borne sulfhydryls explains this disparity.

Studies of the rate at which ethacrynic acid inhibited L-asparagine synthetase showed that, when inhibitor and enzyme were pre-incubated together (in the absence of substrates), maximal inhibition (90% inhibition of L-glutamine utilization at a 2·5 mM concentration of ethacrynic acid) was achieved in approximately 5 min; in the presence of the full complement of substrates, however, the velocity of inhibition of the enzyme by ethacrynic acid was delayed, such that 30 min were required for the production of maximal inhibition (73%).

When attempts were made to determine whether the inhibition produced by ethacrynic acid could be overcome by dialysis, no reversal was seen. This finding points to the stability of the drug enzyme adduct.

Lineweaver Burk plots of the inhibition exerted by ethacrynic acid vs L-glutamine, L-aspartic acid and ammonia produced families of parallel or nearly parallel lines. However, the conclusion that ethacrynic acid is an apparently uncompetitive inhibitor of L-asparagine synthetase must be viewed with caution, in light of the irreversibility described above. Moreover, the rapidity with which such irreversible inhibition is produced probably precluded the measurement of true initial velocities. Tipton [13] has discussed the hazards involved in the interpretation of analogous cases.

Experiments were also carried out to determine whether dithiothreitol could protect against the inhibition produced by ethacrynic acid if it were incubated with L-asparagine synthetase before the addition of inhibitor. For the sake of completeness and in view of the prophylactic effect which substrates often confer on enzymes exposed to thermal and other stresses, L-glutamine, ammonia and ATP were also examined as counter agents to the inhibition exerted by ethacrynic acid (Table 2). ATP $MgCl_2$ (0·01 M each) and dithiothreitol (0·005 M) both partially prevented inhibition by ethacrynic acid (0·005 M), but none of the materials examined could reverse established inhibition, under the circumstances of this study.

Because of the comparatively stable and strong inhibition produced by ethacrynic acid *in vitro* vs tumoral L-asparagine synthetase, attempts were made to inhibit this enzyme *in vivo*. Studies also were carried out on the L-asparagine synthetase of mouse pancreas in the light of a recent survey identifying that organ as one of the principal sites of L-asparagine biosynthesis in mammals [14]. *In vitro*, both of these synthetases are inhibited to a roughly equivalent degree by ethacrynic acid; thus a concentration of the drug of 1×10^{-2} M reduces the utilization of L-glutamine and ammonia by 58 and 70 per cent with the tumoral enzyme, 40 and 69 per cent with its pancreatic counterpart.

In range-finding experiments, all of ten mice given a single intraperitoneal injection of ethacrynic acid at a dose of 200 mg/kg died within 24 hr, while sub-

Table 2. Protection against and reversal of the inhibition of L-asparagine synthetase of L5178Y/AR by ethacrynic acid*

Counter agent	Concn (M)	% Protection		% Reversal	
		Substrate		Substrate	
		L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl
L-Glutamine	1×10^{-2}	11·9	6·1	4·5	1·7
L-Aspartic acid	5×10^{-4}	3·8	0·6	1·8	0
ATP and $MgCl_2$	1×10^{-2}	23·4	16·3	2·1	0
NH_4Cl	1×10^{-1}	5·5	3·5	1·2	0·2
Dithiothreitol	5×10^{-3}	43·3	25·7	5·9	0

* Procedures used for the protection and reversal studies are outlined in Materials and Methods. The synthesis of L-asparagine in the uninhibited reaction was 19·6 and 13·8 nmoles/mg of protein/hr, utilizing L-glutamine and ammonium chloride, respectively, as amide donors.

Table 3. Effect of ethacrynic acid on L-asparagine synthetase activity, *in vivo**

	% Inhibition of L-asparagine synthetase			
	Pancreatic enzyme		L5178Y/AR enzyme	
	Substrates		Substrates	
Dose schedule	L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl
25 mg/kg × 1	57†	58†	0	0
100 mg/kg × 1			27	
25 mg/kg × 5	42†		24	
50 mg/kg × 5	67†	63†	20	0

* Groups of ten BDF_1 mice bearing L5178Y/AR were treated with intraperitoneal injections of ethacrynic acid or saline according to the dose schedule shown in the table. Four hr after the last injection, the mice were killed by neck dislocation, their pancreas and/or tumor removed, homogenized and assayed for L-asparagine synthethase activity as outlined in Materials and Methods.

†$P < 0.01$.

jects given one or two doses of 100 mg/kg convulsed and died within 48 hr of dosing. Fifty and twenty-five mg/kg, per day for 5 days, however, were well tolerated doses and, therefore, were used for further enzymologic and therapeutic studies. Four hr after a single intraperitoneal dose of 100 mg/kg of ethacrynic acid, L-asparagine synthetase activity in tumor of BDF_1 mice bearing L5178Y/AR was depressed only by 27 per cent.§ Four hr after the last of five daily intraperitoneal injections of 50 mg/kg of ethacrynic acid, there was approximately 65 per cent inhibition of pancreatic L-asparagine synthetase activity (Table 3), and this was true irrespective of whether L-glutamine or ammonia was used as substrate. Under the same circumstances, there was only 20 per cent inhibition of the tumoral enzyme with L-glutamine as substrate. This experimental finding was felt to be detrimental to any projected therapeutic use of ethacrynic acid as a tool for converting the L-asparaginase-resistant cell back to the sensitive state; and indeed, when mice were given the drug alone or in combination with L-asparaginase from *E. coli*, no increase in survival was produced (Table 4).

§ Four hr after an intraperitoneal injection of 50 mg/kg of "compound D," no inhibition of L-asparagine synthetase was observed in the tumor (L5178Y/AR) or pancreas of BDF_1 mice.

DISCUSSION

The present series of studies provide evidence that ethacrynic acid is a potent inhibitor *in vitro* of L-asparagine synthetases of murine tumor and pancreas. Presumably, this effect is mediated by the formation of a covalent adduct between crucial sulfhydryl residues on the protein and the methylene side chain of the inhibitor molecule.

In view of the covalent nature of this linkage, it is not surprising that the kinetic patterns of inhibition were substantially irreversible by ordinary dialysis. Of considerable interest is the finding that preincubation of L-asparagine synthetase with ATP and magnesium chloride afforded it considerable protection against attack by ethacrynic acid. This observation would seem to make it likely that the ATP binding site involves or is proximate to a sulfhydryl function.

Unfortunately, the powerful inhibition seen *in vitro* was not so readily demonstrable *in vivo*. Thus, a dose of 50 mg/kg given intraperitoneally for 5 days was

Table 4. Effect of ethacrynic acid and *E. coli* L-asparaginase on survival time of mice bearing leukemia 5178Y*

			L5178Y/AS survival time		L5178Y/AR survival time	
Drug treatment	Dose	Schedule	Mean (days)	Median (days)	Mean (days)	Median (days)
Saline control	0·25 ml		13·8	14	12·2	13
Ethacrynic acid	25 mg/kg	Days 1–10	11·2	12	10·8	11
Ethacrynic acid	50 mg/kg	Days 1–10	5	5	5	5
L-Asparaginase	1000 I.U./kg	Day 3	28	28	14·0	14
Ethacrynic acid + L-asparaginase	25 mg/kg 1000 I.U./kg	Days 1–10 Day 3	12·4	13	9·7	10
Ethacrynic acid + L-asparaginase	50 mg/kg 1000 I.U./kg	Days 1–10 Day 3	12·7	12	5	5

* BDF_1 mice bearing the L5178Y/AS or L5178Y/AR tumor were divided into groups of five mice each. Each group of mice was treated with intraperitoneal injections of ethacrynic acid, *E. coli* L-asparaginase, saline or a combination chemotherapy according to the dose schedule outlined in the table.

required to elicit strong inhibition of pancreatic L-asparagine synthetase activity in the mouse. Five daily doses of 25 mg/kg also inhibited the pancreatic enzyme to a modest degree, but exerted little inhibition on its counterpart in tumor. This finding raises the possibility that the drug penetrates these two tissues to a different degree. Indeed, from a simple anatomical standpoint, a drug injection into the peritoneum of the mouse will quickly bathe the pancreas and very likely penetrate its thin capsule. Although this simple technical fact alone may explain the differential sensitivity of L-asparagine synthetase of pancreas to inhibition by an intraperitoneal dose of ethacrynic acid, further studies by this and other routes, which measure uptake directly, will be needed to settle this point.

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