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EFFECTS OF ASPARAGINE SYNTHETASE INHIBITORS ON ASPARAGINASE RESISTANT TUMORS

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Abstract—Inhibitors of asparagine synthetase have been selected with an asparagine-independent subline of L5178Y lymphoblasts in culture. Five of nineteen asparagine analogs showed selective inhibition of asparagine synthetase. The order of inhibition *in vitro* of cell growth and asparagine synthetase is: L-β-aspartyl methylamide > L-β-aspartyl hydroxamate > L-β-aspartyl hydrazide > D,L-threo-β-methylasparagine > D,L-α-*N*-methylasparagine. The most effective inhibitor of asparagine synthetase, β-aspartyl methylamide, was competitive with glutamine ($K_i = 2 \times 10^{-4}$ M) and ammonia. The β-amide was also relatively resistant to hydrolysis by *Escherichia coli* and *Erwinia carotovora* asparaginases. The β-amide had a plasma half-life of 90 min in mice; was concentrated 5-fold in leukemic cells from ascites fluid; and was metabolized to an α-*N*-acetyl derivative which was excreted in the urine. The compound increased life span from 31 to 79 per cent when administered to mice bearing various asparaginase resistant tumors.

Therapy of malignant disease with L-asparaginase has met with only limited success for several reasons: (1) the majority of neoplasms can synthesize asparagine and are not dependent on an exogenous supply of this amino acid; (2) even in those neoplasms which are initially sensitive to asparaginase, selection of cell lines that are asparagine independent occurs during therapy. These resistant cells have either derepressed formation of asparagine synthetase or have mutated to high constituitive levels of this enzyme; and (3) because of the large molecular weight (135,000), asparaginase is largely restricted to the vascular system. Consequently, tissues such as the liver, which can synthesize asparagine [1], may act as a sanctuary for asparaginase-sensitive tumor cells during therapy [2]. Since the basis of these limitations is the asparagine biosynthetic capability of either the tumor or the host, inhibitors of asparagine biosynthesis may increase the effectiveness and expand the antitumor spectrum of asparaginase therapy.

Asparagine is a product inhibitor ($K_i = 10^{-4}$ M) of asparagine synthetase [3, 4], and also exerts repressional control on the level of enzyme in the cell [5, 6]. The clinical significance of the repressional control mechanism is suggested by the report of Haskell and Canellos [7] that all patients with various forms of leukemia had very low levels of asparagine synthetase prior to asparaginase therapy. After asparaginase therapy, those patients in whose cells synthetase activity increased did not experience a clinical response; conversely, improvement was seen in the patients whose leukemic cells did not increase the enzyme activity. These results suggest that retention of asparaginase sensitivity may be achieved if it is possible to prevent derepression of asparagine synthetase. The fundamental regulatory role of asparagine in controlling its own biosynthesis suggests the possible potential value of asparagine analogs, either to inhibit asparagine synthetase or prevent its derepression or both.

MATERIALS AND METHODS

D,L-β-Aspartyl hydroxamate, D,L-β-aspartyl hydrazide, D-asparagine, and L-β-aspartyl methyl ester were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. The following compounds were prepared in this laboratory by the accompanying literature procedure: D,L-α-*N*-methylasparagine [8], D,L-threo-β-methylasparagine [9], D,L-threo-β-hydroxyasparagine [10], D,L-β-aspartyl semialdehyde [11], L-5-chloro-4-oxo-norvaline [12], L-β-aspartyl chloroethylamide [13], L-5-methylcysteine sulfoximine [14], L-4-oxo-norvaline [15], D,L-threo- and erythro-β-aminoasparagine [15], L-β-aspartyl methylamide [16], L-β-aspartyl isopropylamide [16]], L-β-aspartyl[^{14}C]-methylamide (modified for a 1-m-mole scale using [^{14}C]methylamine hydrochloride, New England Nuclear, Boston, MA) [16] and D,L-β-methylasparagine (modified by the use of Dowex-50, hydrogen form, to free the amino acid from its cupric salt) [17]. L-β-Aspartyl dimethylamide is a new compound prepared by reacting *N*-carbobenzoxy-L-aspartic-β-acid chloride-α-benzyl ester with excess dimethylamine in ether at 0° and deblocking the dimethylamide with hydrogen and platinum on charcoal catalysis in methanol (Calculated: C, 44.99; H, 7.55; N, 17.49. Found: C, 45.26; H, 7.54; N, 17.31). *N*-acetyl-L-β-aspartyl[^{14}C]-methylamide was prepared by incubating the L-β-aspartyl[^{14}C]methylamide in acetic anhydride and separating the products on Dowex 1X8, formate form. Asparaginase from *Escherichia coli* was obtained from Merck, Sharp & Dohme, Rahway, NJ; asparaginase from *Erwinia carotovora* was a gift from Dr. Robert Capizzi, Yale University.

L5178Y sublines with repressible control over

Table 1. Asparagine analog inhibition of L5178Y growth in the presence of asparagine (+ ASN) and absence of asparagine (− ASN) in the growth medium

Compound	Structure	Conc. (mM)	% Inhibition* (− ASN)	(+ ASN)†
D,L-α-*N*-methylasparagine	$HOOCCH(NHCH_3)CH_2CONH_2$	1.0	31	12
D,L-Threo-β-methylasparagine	$HOOCCH(NH_2)CH(CH_3)CONH_2$	1.0	54	30
D,L-Threo-β-hydroxyasparagine	$HOOCCH(NH_2)CH(OH)CONH_2$	1.0	97	0
L-β-Aspartyl methylamide	$HOOCCH(NH_2)CH_2CONHCH_3$	1.0	100	39
		0.1	100	00
D,L-β-Aspartyl hydroxamate	$HOOCCH(NH_2)CH_2CONHOH$	1.0	100	87
		0.2	100	23
D,L-β-Aspartyl hydrazide	$HOOCCH(NH_2)CH_2CONHNH_2$	1.0	100	87
		0.2	96	85
		0.2‡	89‡	45‡
D,L-β-Aspartyl semialdehyde	$HOOCCH(NH_2)CH_2CHO$	0.01	100	(100)
		0.004	20	(22)
L-4-oxo-Norvaline	$HOOCCH(NH_2)CH_2COCH_3$	1.0	100	100
		0.04	75	(66)
		0.04‡	43‡	(59)‡
L-5-Chloro-4-oxo-norvaline	$HOOCCH(NH_2)CH_2COCH_2Cl$	0.01	100	(100)
		0.002	60	(83)
L-β-Aspartyl chloroethylamide	$HOOCCH(NH_2)CH_2CONCH_2CH_2Cl$	1.0	100	100
		0.2	98	92
		0.04‡	20‡	(24)‡

* The number of cells on day 3 of drug-treated culture minus the number of cells on day 0 divided by the number of cells in control culture times 100.

† Media contains 1 mM asparagine except for data in parentheses in which 0.08 mM asparagine was present.

‡ Per cent inhibition on day 2.

asparagine synthetase were obtained as described by Uren *et al.* [5] and the same assay procedures for asparagine synthetase and its repressed-derepressed levels were employed. The kinetics of asparagine synthetase inhibition were performed with between 0 and 2 mM L-β-aspartyl methylamide with 1 and 5 mM glutamine and between 0 and 20 mM β-aspartyl methylamide with 5 and 25 mM ammonium chloride. The metabolism of L-β-aspartyl[^{14}C]methylamide (300 mg/kg) in mice was determined by extracting the tissues with 10% (w/v) trichloroacetic acid. After ether extraction, the acid-soluble extract was chromatographed on a Jeol model 5AH amino acid analyzer. The long column elution was collected in 1-ml fractions and counted. The *N*-acetyl-β-aspartyl[^{14}C]-methylamide metabolite eluted at fraction 14 (the end of the void volume) and unmodified β-aspartyl[^{14}C]-methylamide at fraction 32 corresponding to the elution position of threonine. The concentration of asparagine in acid-soluble extracts of plasma and the livers of drug-treated L5178Y leukemic mice was measured with the amino acid analyzer using lithium citrate buffer systems.

RESULTS

A previous report from this laboratory [5] described a L5178Y subline which grew either in the presence or absence of asparagine. In the absence of asparagine, this subline grew with a characteristic lag in its rate of growth which correlated with the time necessary to derepress asparagine synthetase. Since in the absence of asparagine the synthetase activity is rate limiting for growth, inhibitors of the enzyme would cause a proportional inhibition of cell growth. In the presence of asparagine, cell growth is not dependent on asparagine synthetase and inhibition of the enzyme should not inhibit the rate of cell growth.

Nineteen asparagine analogs were tested in this cell growth assay (Table 1). Compounds without inhibitory effects at 10^{-3} M include: D-asparagine, D,L-α-methylasparagine, D,L-erythro-β-amino asparagine, D,L-threo-β-amino asparagine, L-β-aspartyl isopropylamide, L-β-aspartyl ethylamide, L-β-aspartyl dimethylamide, L-β-aspartyl methylester, and L-*S*-methylcysteine sulfoximine. Compounds which inhibited growth in the absence but not in the presence of asparagine follow in the order of decreasing effectiveness: L-β-aspartyl methylamide > D,L-β-aspartyl hydroxamate > D,L-β-aspartyl hydrazide > D,L-threo-β-hydroxyasparagine > D,L-threo-β-methylasparagine > and D,L-α-*N*-methylasparagine.

The effects of these compounds on asparagine synthetase are shown in Table 2. Under the assay conditions where asparagine, a product inhibitor, causes 91 per cent inhibition, the order of decreasing effectiveness toward asparagine synthesis inhibition is: L-β-aspartyl methylamide, D,L-β-aspartyl hydroxamate, D,L-β-aspartyl hydrazide, D,L-threo-β-methylasparagine and D,L-α-*N*-methyl-asparagine. This order is similar to that for cell growth inhibition except for D,L-threo-β-hydroxyasparagine which, unlike the other compounds, inhibits the charging of asparaginyl-tRNA but not aspartyl-tRNA (C. Griffin, personal communication). All of the active compounds also reduced the degree of derepression of asparagine synthetase when the cells were grown in the absence of asparagine in proportion to their inhibition of cell growth. An L5178Y subline which expresses asparagine synthetase activity in a constitutive manner was also inhibited by the above compounds. The type and magnitude of the inhibition by the most potent inhibi-

Table 2. Inhibition of asparagine synthetase *in vitro*

Addition	Activity* (nmoles/hr)	% Inhibition
None	4.43	
L-Asparagine	0.38	91
L-β-Aspartyl methylamide	1.46	67
D,L-β-Aspartyl hydroxamate	2.72	39
D,L-β-Aspartyl hydrazide	3.81	14
D,L-Threo-β-methyl-asparagine	4.03	9
D,L-α-*N*-methyl-asparagine	4.25	4
D,L-Threo-β-hydroxy-asparagine	4.50	0

* Assay contains 1 mM glutamine, and was performed as described by Uren *et al.* [5] with all inhibitors present at a concentration of 1 mM.

tor, L-β-aspartyl methylamide, are presented in Fig. 1. L-β-Aspartyl methylamide showed competitive inhibition with glutamine with a K_i of 2×10^{-4} M; the compound also competitively inhibited the utilization of ammonia with a K_i of 6×10^{-4} M.

To determine whether β-aspartyl[^{14}C]methylamide was incorporated into protein, the analog (1×10^{-4} M, 7.3×10^6 cpm/ml) was incubated with L5178Y cells (4.7×10^6/ml) in Fischer's medium for 2 hr. Comparisons with a control experiment, in which an equivalent amount of [^{14}C]asparagine (1×10^{-4} M, 7×10^6 cpm/ml) was incubated, demonstrated that the methylamide compound was incorporated into acid-insoluble counts at less than 2.0 per cent the rate of asparagine. It seems unlikely that the mechanism of inhibition of cell growth is related to this low level of incorporation into acid-insoluble material.

Considerations for therapy in vivo

Since asparagine prevented the inhibition of tumor cell growth in culture caused by the asparagine analogs, therapy *in vivo* should be evaluated under conditions of asparagine depletion as can be achieved by administering the enzyme L-asparaginase. Since they might also be substrates, the stability of the three most potent inhibitors of tumor growth in the presence of *E. coli* and *Erwina carotovora* asparaginase was examined (Table 3). The most potent inhibitor of tumor growth, β-aspartyl methylamide, was also the most resistant to hydrolysis by either the *E. coli* or *Erwina* asparaginase. Although this compound was a poor substrate for bacterial asparaginases, it proved to be a good inhibitor of the *E. coli* enzyme with a K_i of 3×10^{-5} M. These results were consistent with the K_m for β-aspartyl methylamide as a substrate of 3×10^{-5} M reported by Herrmann *et al.* [18].

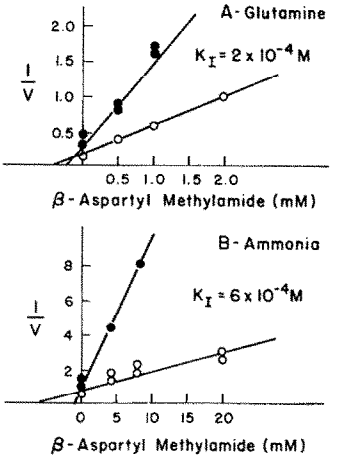

Fig. 1. Dixon plots for the inhibition of asparagine synthetase by β-aspartyl methylamide. Panel A: glutamine concentrations of 1 mM (—●—) and 5 mM (—○—), respectively; panel B: 5 mM (—●—) and 25 mM (—○—) ammonium chloride replacing the glutamine as substrate. The procedures are described in Materials and Methods.

The inhibitory effects of β-aspartyl methylamide in tissue culture, as a function of concentration and time prior to its removal, are shown in Fig. 2. These data show that, within the time periods examined, 2 mM β-aspartyl methylamide present for 1 day or 0.1 mM present for 2 days will completely prevent subsequent cell growth. These results suggest the lower limits of concentration and time which therapy *in vivo* should seek to approximate.

Properties in vivo *of β-aspartyl methylamide*

The plasma clearance of β-aspartyl methylamide in mice is shown in Fig. 3. The plasma half-life (90 min) was much longer than that for asparagine and was not decreased if the animal was pretreated with *E. coli* asparaginase.

Using the ^{14}C-labeled β-aspartyl methylamide, the tissue distribution 1 hr after injection into a leukemic mouse is shown in Table 4. It was observed that, after correcting for the percentage of metabolites present as determined with an amino acid analyzer, a 2-fold concentration of the compound had occurred in the liver compared to plasma and a 5-fold concentration in tumor cells compared to ascites fluid. A

Table 3. Analog hydrolysis by asparaginase

Substrate	*E. coli**		*Erwina carotovora**	
	(μmoles/min)	(%)	(μmoles/min)	(%)
L-Asparagine	10.1	100	79	100
D,L-β-Aspartyl hydroxamate	4.1	40	18	23
D,L-β-Aspartyl hydrazide	0.22	2.2	2.9	3.7
L-β-Aspartyl methylamide	0.16	1.6	1.3	1.7

* Incubation conditions and assay for the product of the reaction, aspartic acid, were those described for a coupled enzyme assay described by Cooney *et al.* [19]. All substrates were at a concentration of 0.2 mM.

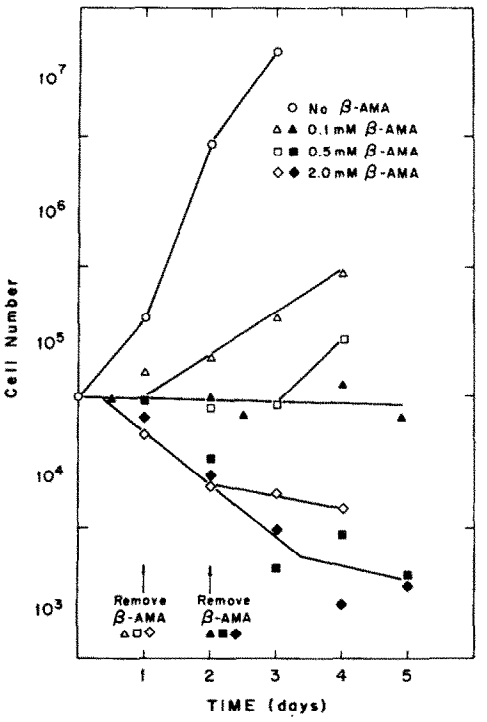

Fig. 2. Effect of β-aspartyl methylamide (β-AMA) on the growth of an asparagine-independent L5178Y subline. Cells were grown in Fischer's media lacking asparagine with varying concentrations of β-AMA: (—O—) none; (—△—) 0.1 mM; (—□—) 0.5 mM; and (—◇—) 2.0 mM. After 1 day (open symbols) or 2 days (closed symbols), the cells were centrifuged and resuspended in fresh media without inhibitor. The cell number was determined with a Coulter counter on days 1–5.

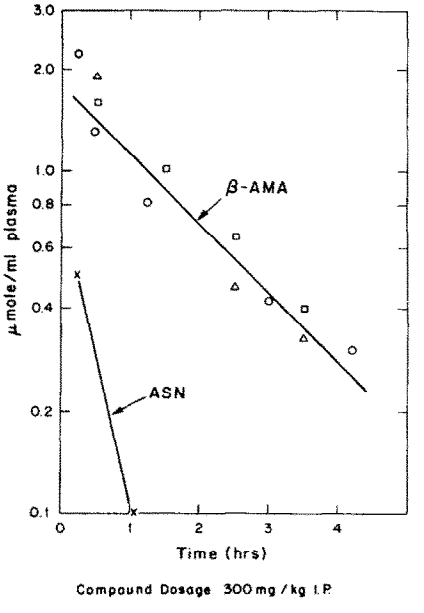

Fig. 3. Plasma half-life of β-aspartyl methylamide (β-AMA) and asparagine (ASN) in BDF mice. The compounds were administered at 300 mg/kg, i.p. Plasma was prepared from blood collected by orbital bleeding. The concentration of the methylamide was measured as radioactivity in experiments designated by the symbols ×, △ and □. In another experiment (—O—), the plasma concentration of the analog was measured as the increment in the concentration of aspartic acid after treatment with 200 units asparaginase/ml by a coupled enzyme assay described by Cooney *et al.* [19].

major hepatic metabolite eluted in the acidic region on the amino acid analyzer and was also present in the urine; unmetabolized β-aspartyl methylamide was not excreted under these conditions.

The identity of the metabolite was determined by the following experiments. Samples of mouse urine taken 5 hr after the injection of 300 mg [^{14}C]β-aspartyl methylamide/kg contained the radioactive metabolite at a concentration of 100 mM. Despite the high concentration of metabolite, no ninhydrin reactive material above levels found in normal mouse urine was observed. Acid hydrolysis of this urine produced large amounts of aspartic acid. These results were consistent with conjugation of the α-amino function of β-aspartyl methylamide. Treatment of the urine with 1000 units of hog kidney acylase regenerated the unmodified β-aspartyl methylamide, suggesting *N*-acetylation as the major route of metabolism *in vivo*. Samples of synthetic *N*-acetyl-β[^{14}C]aspartyl methylamide showed the identical chromatographic mobility as the major urinary and liver metabolite. The *N*-acetylated compound was less than $\frac{1}{20}$ as active as an inhibitor of L5178Y subline in culture as β-aspartyl methylamide.

Plasma and liver levels of asparagine were measured in leukemic mice after intraperitoneal treatment with asparaginase (200 units/kg/day), β-aspartyl methylamide (300 mg/kg twice daily), and the combination. After 3–6 days of combination therapy, the

Table 4. [^{14}C]β-aspartyl methylamide (β-AMA) tissue distribution*

Tissue (fluid)	Total radioactivity (cpm/g or ml)	Per cent β-AMA	Ratio: β-AMA tissue / β-AMA fluid
Ascites fluid	63,700	90	
Ascites L5178Y cells	330,000	90	5.2
Plasma	13,000	90	
Liver	47,000	50	2.0
Urine	1,080,000	0	

* Mice previously inoculated with 10^6 L5178Y cells (7 days) were given an 0.5 ml i.p. injection of [^{14}C]β-aspartyl methylamide (15 mg/kg, 0.5 μCi). After 1 hr the mice were sacrificed and acid-soluble extracts of tissues and fluids were prepared and analyzed as described in Materials and Methods.

Table 5. Antitumor activity of β-aspartyl methylamide*

Treatment		Saline		β-Aspartyl methylamide		
Tumor	Host	Weight change† (%)	Mean survival time (days)	Weight change† (%)	Mean survival time (days)	Per cent ILS‡
L1210	BDF	+15	7.2 ± 0.4	0	10.6 ± 0.4	47
S180	CDI	+14	10.8 ± 1.1	+6	14.2 ± 0.8	31
AD755	AKD	+12	12.2 ± 1.0	+1	21.8 ± 1.0	79

* Groups of five mice were given intraperitoneal injections of 10^6 tumor cells and 0.1 ml of sterile saline, or analog (300 mg/kg) solution twice daily on days 2–5.

† Per cent weight loss or gain (weight on day 5/weight on day 1 × 100).

‡ Per cent ILS (increase in life span) treated ÷ control × 100 − 100.

liver concentration of asparagine was 25% of normal and the plasma level was undetectable, an effect equivalent to that of asparaginase alone. Treatment with β-aspartyl methylamide alone did not reduce the asparagine concentration in the liver or the plasma. This lack of an effect of β-aspartyl methylamide on the liver asparagine synthetase may relate to the rapid metabolism of the compound by the liver. The effects of the compound on asparagine synthetase *in vitro* in the liver could not be measured in crude homogenates because of the rapid metabolism of the [^{14}C]aspartic acid and the [^{14}C]asparagine formed.

The antitumor activity of β-aspartyl methylamide is shown in Table 5. When the compound was administered at high doses (300 mg/kg) twice daily, a significant increase in the life span (31–79 per cent) of mice bearing three asparaginase resistant tumors was observed. When the compound was combined with asparaginase, toxicity was enhanced with a decrease in the survival of the mice compared to results obtained with the compound alone. The asparaginase-sensitive L5178Y tumor did not respond significantly better when the compound was combined with asparaginase as compared to asparaginase alone.

DISCUSSION

The ability of asparagine analogs to inhibit the growth of an L5178Y subline in culture only in the absence of asparagine proved to be a useful screen for compounds which selectively inhibit asparagine synthetase. All but one of the compounds which inhibited cell growth only in the absence of asparagine inhibited asparagine synthetase to a degree which correlated with the degree of inhibition of cell growth. The only exception, β-hydroxy-asparagine, appears to inhibit the charging of asparaginyl-tRNA but not aspartyl-tRNA. The structure–activity relationships presented in Tables 1 and 2 suggested that there exists on asparagine synthetase some bulk tolerance in the threo position of the β-carbon of asparagine and that one proton in the amide nitrogen can be replaced with a group smaller than ethyl. D,L-β-Aspartyl hydroxamate was previously reported to inhibit asparagine synthetase from a human tumor [20]. Another previously reported inhibitor of the enzyme, 5-chloro-4-oxo-norvaline [3], does inhibit cell growth, but the inhibition was not nullified by the addition of asparagine to the media. This result was consistent with the ability of the compound to inhibit other glutamine requiring enzymes [12], and indicates the selectivity of the cell culture assay reported here. L-*S*-methyl-cysteine sulfoximine does not inhibit cell growth although its next higher homolog, methionine sulfoximine, has been reported to be a potent inhibitor of glutamine synthetase [21].

The three most effective inhibitors of asparagine synthetase, β-aspartyl methylamide, β-aspartyl hydroxamate and β-aspartyl hydrazide, when added to a cell culture deprived of asparagine at a level which slows cell growth, would prevent the derepression of asparagine synthetase levels. Whether these compounds function as corepressors or block the derepression by inhibiting general protein synthesis consequent to depriving the cells of asparagine remains unanswered. Although the results were consistent with a corepressor function, their selective action on only asparagine synthetase has not been demonstrated. Precedence for such selectivity has been obtained by Norton and Chen [22], who have shown that in a strain of *Lactobacillus arabinosus*, which will derepress both its asparagine and glutamine synthetases, β-aspartyl hydroxamate will selectively prevent the derepression of only the asparagine synthetase.

The most effective cell growth inhibitor, β-aspartyl methylamide, was also the most effective inhibitor of asparagine synthetase and most resistant to hydrolysis by bacterial asparaginases. This resistance to hydrolysis was reflected *in vivo* in the unchanged plasma half-life of the compound when the animal was pretreated with a therapeutic level of asparaginase. The longer plasma half-life compared to asparagine also reflects an increased stability toward liver asparaginase. Although the compound was an effective inhibitor *in vitro* of asparagine synthetase, *in vivo* it did not add to the reduction in the concentration of asparagine in liver or plasma caused by injecting *E. coli* asparaginase. Similarly the increase in survival of asparaginase-sensitive L5178Y leukemic mice was not potentiated by simultaneous treatment with the analog.

The rapid metabolism *in vivo* to *N*-acetyl-β-aspartyl methylamide and excretion of this material required the administration of at least 300 mg/kg of the compound twice daily to maintain an average plasma level of the analog of about 0.1 mM. A 4-day therapy schedule at this dosage should be sufficient to prevent growth of leukemic cells as judged by the cell culture data in Fig. 2. This therapy regimen significantly increased the life of mice bearing various asparaginase

resistant tumors (Table 5). The lack of synergistic responses with the combined therapy is probably related to the fact that the asparagine analog is a better inhibitor of asparaginase ($K_i = 3 \times 10^{-5}$ M) than asparagine synthetase ($K_i = 2 \times 10^{-4}$ M). Thus, the asparaginase activity is neutralized by the simultaneous presence of the analog at a level of 0.1 mM. Nevertheless, these increases in survival support the proposal that inhibitors of asparagine synthetase will show antitumor efficacy over a broader spectrum of tumors than those which will respond to asparaginase alone. It is our belief that more potent inhibitors of asparagine synthetase which do not inhibit nor are hydrolyzed by asparaginase will improve survival beyond that observed in this work.

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