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Antitumor Activity of DL-*threo*-β-Fluoroasparagine against Human Leukemia Cells in Culture and L1210 Cells in DBA Mice[1]

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ABSTRACT

DL-*threo*-β-Fluoroasparagine (DL-*threo*-β-F-Asn) inhibited the growth of murine leukemia L1210 cells and three human leukemia cell lines in culture. Fifty % inhibiting dose values ranged between 30 and 50 μM DL-*threo*-β-F-Asn. L1210 cells were not sensitive to DL-*erythro*-β-fluoroasparagine, DL-*threo*-β-fluoroaspartic acid, or DL-*erythro*-β-fluoroaspartic acid at 300 μM, the highest dose studied. The antileukemia activity of DL-*threo*-β-F-Asn was studied in further detail using the L1210 model system. Inhibition of growth in culture was prevented by L-asparagine but not by D-asparagine. Inhibition of growth of L1210 cells incubated for 40 hr in the presence of 300 μM DL-*threo*-β-F-Asn was reversed after DL-*threo*-β-F-Asn removal. Treatment for longer periods of time resulted in cell lysis. DL-*threo*-β-F-Asn at doses of 250 mg/kg increased life span in mice bearing L1210 tumors by 60%. These results demonstrate the chemotherapeutic potential of the amino acid analogue DL-*threo*-β-F-Asn.

INTRODUCTION

An important route for the development of pharmacologically active compounds is the synthesis of analogues of substrates that have a chemical reactivity different from that of their natural counterparts but can nevertheless compete effectively for the same recognition sites. Much of the success and present emphasis in the development of antitumor agents has come from the synthesis of purine and pyrimidine analogues. In contrast, despite the observations that certain tumors have unique nutritional requirements for amino acids such as asparagine (2, 5, 8, 10) and cysteine (16) and thus might be appropriate targets for analogues of these amino acids, there have been relatively few amino acid analogues that have been developed as antitumor agents. L-β-aspartyl-methyl amide, an asparagine analogue (15), and canavanine, an arginine analogue (4), are 2 examples of amino acid analogues that are active in animals bearing the L1210 murine leukemia. In order to develop other amino acid analogues with chemotherapeutic potential, we synthesized and studied DL-*threo*-β-fluoroaspartate and DL-*threo*-β-fluoroasparagine (14). These amino acid analogues were potent and selective cytotoxic agents for several types of mammalian cells in culture. Both of these amino acid analogues can become incorporated into protein, and this property appears to be important in their mode of action (6, 11, 14). DL-*threo*-β-F-Asn[4] can replace L-Asn in protein but cannot serve as a site for *N*-linked glycosylation (6, 11). Thus, asparagine-linked glycosylation of proteins is inhibited in cells treated with DL-*threo*-β-F-Asn. A preliminary communication has reported that DL-*threo*-β-F-Asn is active against L1210 cells in culture (3). The present investigation was designed to evaluate the chemotherapeutic potential of DL-*threo*-β-F-Asn by examining its ability to inhibit the growth of several human leukemia cells in culture and to increase the life span of mice bearing the L1210 tumor.

MATERIALS AND METHODS

Amino Acids. DL-*threo*-β-F-Asn, DL-*erythro*-β-F-Asn, DL-*threo*-β-F-Asp·HCl and DL-*erythro*-β-F-Asp·HCl were synthesized from their corresponding β-OH analogues as described previously (11, 14). Several preparations of DL-*threo*-β-F-Asn were required for the *in vivo* studies. The authenticity and purity of each preparation was monitored by nuclear magnetic resonance (14) and high-voltage electrophoresis at pH 1.9 and 8.9 (14). Electrophoretograms loaded with 300 to 500 nmol of DL-*threo*-β-F-Asn showed traces of the starting material, DL-*threo*-β-OH-Asn, which was estimated to be present at <1 nmol. No other ninhydrin-positive material was detected. Hydrolysis of DL-*threo*-β-F-Asn in 2 N HCl at 37° for 2 days was complete and resulted in a single ninhydrin-positive species which comigrated with authentic DL-*threo*-β-F-Asp at pH 1.9 (14). The purity of the preparations of DL-*threo*-β-F-Asn used *in vivo* was also monitored by amino acid analysis. The Durrum D-500 (System I) and Beckman 6300 (System II) systems using sodium citrate buffers and programmed for the analysis of protein hydrolyzates were used. Amino acids were applied in pH 3.2 buffer and emerged as single symmetric peaks with the following retention times (min): System I DL-*threo*-β-F-Asp, 5.1; DL-*threo*-β-F-Asn, 7.9; L-Asp, 12.6; System II: cysteic acid, 2.37; DL-*threo*-β-F-Asn, 4.52; DL-*erythro*-β-F-Asn, 5.20; L-Asp, 7.85. A ninhydrin-positive impurity having a similar color yield to DL-*threo*-β-F-Asn and present at 0.3% would be detectable under the conditions used. L-Asn·H_2O (Sigma), D-Asn·H_2O (Sigma), and L-Asp (Reliable Reagents) were used without further purification.

Cell Culture. L1210 lymphocyte murine leukemia cells (American Type Culture Collection, CCL 219) were obtained from Dr. Pierre Major, Dana-Farber Cancer Institute, Boston, MA. These cells were grown in Dulbecco's modified Eagle's medium [glucose (4.5 g/liter):penicillin (100 units/ml):streptomycin (100 μg/ml):2 mM L-glutamine:70 μM 2-mercaptoethanol] supplemented with 10% fetal bovine serum. The cells were passed every 3 days by 20-fold dilution into fresh medium at a density of 1×10^5 cells/ml. IM-9, human lymphoblast cells (ATCC, CCL 159) and RPMI 8226 (ATCC, CCL 155), human myeloma cells, were grown in RPMI 1640 medium supplemented with 7.5% horse serum and 2.5% fetal bovine serum. CCRF-SB, human lymphoblastic leukemia cells (ATCC, CCL 120) were grown in Eagle's minimal essential medium supplemented with 5% horse serum and 5% fetal bovine serum. Culture media and sera were obtained from Grand Island Biological Co.

Stock solutions of amino acids were prepared as 20- to 100-fold concentrates in the appropriate medium. These solutions were sterilized by filtration. L-Asp solutions were neutralized with 0.2 N sodium hydroxide. Cell growth was measured by cell count using a hemocytometer. Cell viability was determined by treating the cells with 0.2% trypan blue

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[4] The abbreviations used are: DL-*threo*-β-F-Asn, DL-*threo*-β-fluoroasparagine = DL-*threo*-3-fluoroasparagine; DL-*threo*-β-F-Asp, DL-*threo*-β-fluoroaspartic acid = DL-*threo*-3-fluoroaspartic acid; L-Asp, L-aspartic acid; L-Asn, L-asparagine; DL-*threo*-β-OH-Asn, DL-*threo*-β-hydroxyasparagine = DL-*threo*-3-hydroxyasparagine.

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for 3 min and then monitoring for dye exclusion.

Animal Studies. Female DBA/2J mice (The Jackson Laboratory), 8 to 12 weeks old and weighing 18 to 22 g, received an i.p. injection of 1×10^5 L1210 cells in 0.3 ml of isotonic saline on Day 0. The injection L1210 cells were maintained in culture as described above. DL-*threo*-β-F-Asn solutions in 0.85% NaCl and neutralized with Na_2CO_3, where indicated, were made fresh daily, kept at 4°, and were filter sterilized before use. DL-*threo*-β-F-Asn was administered by an i.p. injection in a volume of 0.3 to 0.4 ml/animal. Animals were monitored twice daily.

RESULTS

Cell Culture Studies. Our initial studies examined the ability of DL-*threo*-β-F-Asn to inhibit the growth of murine leukemia L1210 and several human leukemia cells in culture. A 50% inhibition of L1210 cell growth was achieved at 45 μM DL-*threo*-β-F-Asn (Chart 1). At 300 μM, the highest dose studied, DL-*threo*-β-OH-Asn and DL-*erythro*-β-F-Asn showed a 20 to 25% inhibition of growth, while DL-*threo*-β-F-Asp and DL-*erythro*-β-F-Asp showed no significant activity. The low potency of DL-*threo*-β-F-Asp to inhibit the growth of L1210 cells compared to DL-*threo*-β-F-Asn excludes the possibility that the growth-inhibitory properties of DL-*threo*-β-F-Asn are due to its extracellular conversion to DL-*threo*-β-F-Asp via a medium-catalyzed hydrolysis reaction.

The effects of DL-*threo*-β-F-Asn on the growth of 3 human leukemia cell lines in culture were determined (Chart 2). The 3

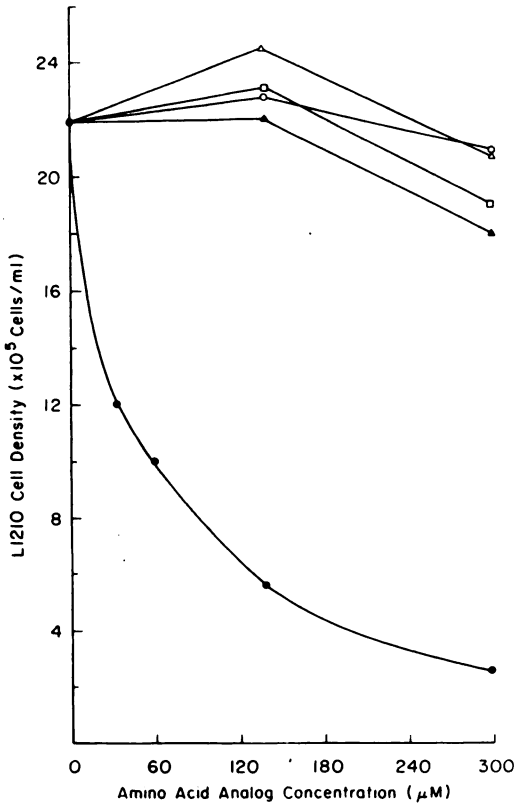

Chart 1. Actions of DL-β-amino acid analogues on L1210 cell growth. An L1210 cell suspension (1×10^5 cells/ml) in Dulbecco's modified Eagle's medium was added to 35- x 10-mm dishes (1.5 ml/dish). After a 1-hr incubation period, each culture was treated with one of the following amino acid analogues: ●, DL-*threo*-β-F-Asn; ▲, DL-*erythro*-β-F-Asn; □, DL-*threo*-β-OH-Asn; O, DL-*threo*-β-F-Asp·HCl; and △, DL-*erythro*-β-F-Asp·HCl; and incubation was continued for 45 hr. After this period, the cell number was counted. The values shown represent means of duplicate dishes with a maximum range equal to ±15%.

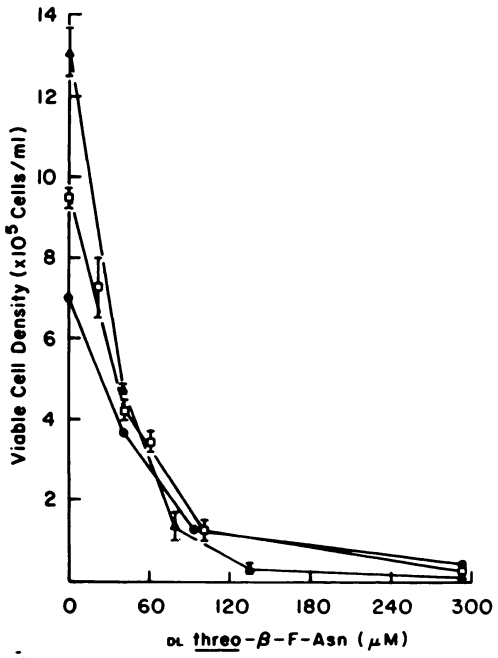

Chart 2. Growth inhibition of human leukemia cells by DL-*threo*-β-F-Asn. RPMI 8226, CCRF-SB, and IM-9 cells were seeded in 35- x 10-mm dishes containing 1.5 ml of RPMI 1640 medium depleted in L-Asp and L-Asn for RPMI 8226 and IM-9 cells and minimal essential medium for CCRF-SB cells at an initial density of 2.4×10^5 cells/ml (>95% viability). Two hr after seeding, the cells were treated with various concentrations of DL-*threo*-β-F-Asn. After 3.5 days, the cells were counted, and the viability was determined. ▲ IM-9; ●, CCRF-SB; □, RPMI 8226. *Bars*, S.D.

cell lines are similarly sensitive to the growth-inhibitory effects of DL-*threo*-β-F-Asn. A 50% inhibition of cell growth was achieved at approximately 40 μM DL-*threo*-β-F-Asn. At concentrations greater than 75 μM, the viable cell densities were below the initial seeding densities. These results demonstrate that L1210 cells and the 3 human cell lines respond similarly to DL-*threo*-β-F-Asn. None of these lines is asparagine-requiring, since they can be grown without the addition of L-Asn.

The growth-inhibitory activity of DL-*threo*-β-F-Asn was characterized further using the L1210 model system. L-Asn but not D-Asn prevented the growth inhibition of L1210 cells by DL-*threo*-β-F-Asn (Chart 3). L-Asp at 1 mM was not effective in reducing the growth inhibition by DL-*threo*-β-F-Asn. Growth of L1210 cells incubated for 40 hr in the presence of 300 μM DL-*threo*-β-F-Asn was inhibited by 90%. This inhibition of cell growth was reversible after removal of DL-*threo*-β-F-Asn (Chart 4). In a separate experiment, cell viability was measured by the ability of L1210 cells to exclude trypan blue as a function of incubation time with DL-*threo*-β-F-Asn. After 40 hr of growth in the presence of 300 μM DL-*threo*-β-F-Asn, 98% of the treated cells excluded trypan blue. This result is consistent with an initial reversible action of DL-*threo*-β-F-Asn. However, after an additional 32 hr of incubation, some cell lysis was apparent, and only 45% of the treated (100% of the untreated cells) cells excluded trypan blue. Thus, the growth inhibition of L1210 cells in culture by DL-*threo*-β-F-Asn results from an initial reversible cytostatic effect, followed by a cytotoxic effect, which eventually leads to cell lysis.

Animal Experiments. Since DL-*threo*-β-F-Asn inhibits the growth of L1210 cells in culture, we undertook experiments to explore the actions of DL-*threo*-β-F-Asn on DBA/2 mice bearing the L1210 tumor. Several experiments were performed in which

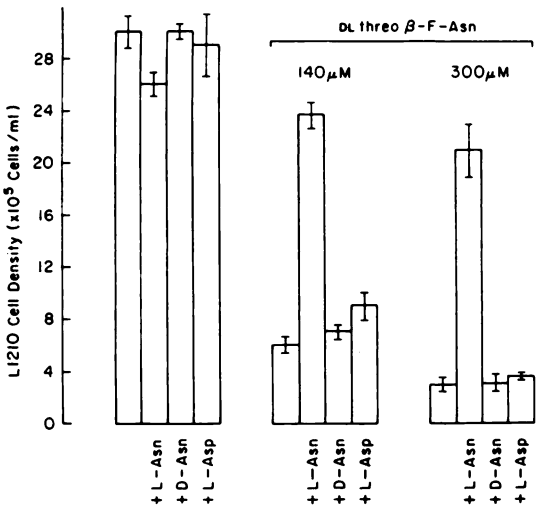

Chart 3. Prevention of DL-*threo*-β-F-Asn growth inhibition by L-Asn. An L1210 cell suspension (1×10^5 cells/ml) in Dulbecco's modified Eagle's medium was added to 35- x 10-mm dishes (1.5 ml/dish). After 1-hr incubation period, the dishes were divided into 3 groups, and each group was treated without or with DL-*threo*-β-F-Asn at 140 or 300 μM final concentrations. Within each group, cultures were treated with 1 mM L-Asn, D-Asn, or L-Asp. After a 48-hr incubation period, the cell number was counted. *Bars*, mean of duplicate dishes; *brackets*, range.

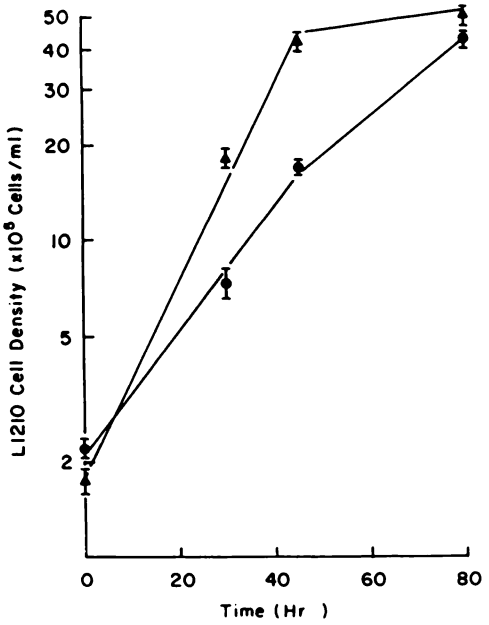

Chart 4. Growth of L1210 cells following DL-*threo*-β-F-Asn removal. An L1210 cell suspension (1.5×10^5 cells/ml) in Dulbecco's modified Eagle's medium was divided equally; one half was treated with DL-*threo*-β-F-Asn at a final concentration of 300 μM. The other half served as an untreated control. These cell suspensions were seeded into 35- x 10-mm dishes (2.2×10^6 cells/dish) and incubated for 40 hr. The cell numbers after this period were 1.5×10^6 cells/ml for the controls and 2.9×10^5 cells/ml for the treated group. The cell suspensions from 5 control dishes and 15 treated dishes were pooled separately into sterile centrifuge tubes and diluted to 20 ml with sterile Dulbecco's modified Eagle's medium, and the cells were pelleted by centrifugation. The supernatant medium was removed, and the cells were resuspended in Dulbecco's modified Eagle's medium to a density of 2×10^6 cells/ml. These cell suspensions were divided into 35- x 10-mm dishes (3×10^5 cells/dish) and incubated for 80 hr. At the time intervals indicated, 2 dishes from each group were used to determine cell number. ▲, untreated; ●, treated. *Bars*, S.D.

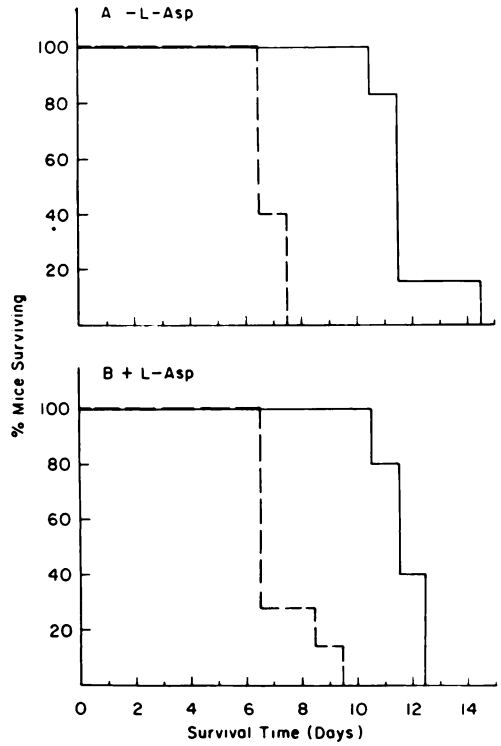

Chart 5. Survival curves for untreated and treated mice bearing L1210 cells. Twenty-four hr after tumor cell injection, treatment with DL-*threo*-β-F-Asn was initiated with 2 injections (0.4 ml, i.p.) per day (10 a.m., 3 p.m.), each containing 250 mg/kg. Treatment was continued for 12 days. Neutralized saline for untreated controls and neutralized DL-*threo*-β-F-Asn solutions were used in this experiment (see "Materials and Methods). *A*, treatment with DL-*threo*-β-F-Asn only. The untreated control and treated groups each contained 6 animals. *B*, treatment with DL-*threo*-β-F-Asn + L-Asp (1500 mg/kg). The untreated control group contained 7 animals; the treated group contained 5 animals. In *A*, the mean survival times were: 7.4 ± 0.5 days (S.D.) for the untreated control group and 12.3 ± 1.4 days for the treated group. In *B*, the mean survival times were: 7.7 ± 1.3 days for the untreated control group and 12.2 ± 1.0 for the treated group. ——, treated group; - - -, untreated group.

the treatment conditions were varied. A dose of DL-*threo*-β-F-Asn (250 mg/kg) given twice daily proved optimal. An experiment using this dose and schedule, in which the median survival time was increased 66% from 7.4 ± 0.5 (S.D.) to 12.3 ± 1.4 days is shown in Chart 5. At a dose of 125 mg/kg, DL-*threo*-β-F-Asn increased the median survival time by 30%, from 7.3 ± 0.7 to 9.5 ± 0.5 days. Other experiments using shorter treatment schedules resulted in insignificant increases in life span. Thus, at a dose of 250 mg/kg given twice daily on Days 1, 2, and 3 and at a dose of 125 mg/kg given twice daily on Days 1, 2, 3, and 4, increases in life span of 10 and 13%, respectively, were observed. The need for continued treatment beyond Day 4 may be due to the relatively long time [2 to 3 days (see above)] required for irreversible inhibition of L1210 cell growth to occur by DL-*threo*-β-F-Asn treatment and the relatively short half-life (less than 2 hr) of DL-*threo*-β-F-Asn in plasma.[5]

L-Asp at 1 mM was not effective in reducing the growth inhibition of L1210 cells by DL-*threo*-β-F-Asn (Chart 3). However, our previous results (14) have demonstrated that, for some cells, L-Asp can prevent the cytotoxic effects of DL-*threo*-β-F-Asn. If there is some general toxicity toward normal cells caused by DL-

[5] Our preliminary studies have shown that, in DBA/2 mice given injections i.p. with DL-*threo*-β-F-Asn (125 mg/kg), no DL-*threo*-β-F-Asn could be detected by amino acid analysis in the plasma 3 hr after administration.

threo-β-F-Asn that can be prevented by L-Asp, then a combination of L-Asp and DL-*threo*-β-F-Asn might prove to be more effective than is DL-*threo*-β-F-Asn alone against the L1210 tumor *in vivo*. The results of such an experiment are shown in Chart 5. The L-Asp + DL-*threo*-β-F-Asn combination proved to be no more effective than was DL-*threo*-β-F-Asn alone in increasing the median survival time.

The toxicity of DL-*threo*-β-F-Asn in non-tumor-bearing DBA/2 mice was evaluated. At the therapeutic levels of treatment (250 mg/kg, twice daily), in one experiment with 8 animals, 1 animal died on Day 10 of treatment and, in another experiment with 4 animals, 2 died on Day 10 of treatment. Treatment of the surviving animals was terminated on Day 11 in the first experiment and on Day 12 in the second experiment. All surviving animals lived for the duration of the experiment (30 days after treatment was stopped). In 2 experiments in which groups of 5 animals were used, a dose of 500 mg/kg given twice daily either alone or in combination with L-Asp (2.5 g/kg) resulted in all of the animals dying on Days 2 and 3 of treatment.

DISCUSSION

The most important finding in this study is the significant *in vivo* activity of DL-*threo*-β-F-Asn against the L1210 tumor. These findings imply that DL-*threo*-β-F-Asn is acting on the L1210 tumor cells with a certain degree of selectivity that could arise for one or more of the following reasons: (*a*) preferential uptake of the amino acid analogue by the tumor cells; (*b*) lower intracellular levels of asparagine, the competing natural amino acid, within the tumor cells, which could arise from a unique nutritional requirement for asparagine or from a metabolic imbalance that results in depletion of the free asparagine pool; (*c*) a higher turnover rate of proteins within the tumor cells as compared to other cells, whose activity and function are particularly sensitive to the incorporated analogue; or (*d*) metabolic conversion of the amino acid analogue, that could give rise to lower rates of detoxification or higher rates of toxic metabolite production within the tumor cells as compared to other cells.

The L1210 tumor does not have a nutritional requirement for L-Asn, as do L5178Y and Jensen sarcoma cells, and, thus, it does not respond to asparaginase therapy (7). However, it has been reported recently (12)[6] that the early ascitic form of L1210 (3 days after i.p. implantation) does not have detectable levels of L-Asn in its free amino acid pool. In contrast, the 5-day L1210 tumor has levels of L-Asn in its free amino acid pool equal to 40 μM, the level of L-Asn in the plasma. Thus, the quantitative differences in L-Asn metabolism for the early ascitic form of L1210 as compared to the later form may selectively predispose the early form to the cytostatic and cytotoxic actions of DL-*threo*-β-F-Asn.

The results in this report establish the chemotherapeutic potential of DL-*threo*-β-F-Asn. Therefore, an understanding of the molecular basis for the antileukemia activity of this compound now becomes important. Although, at the present time, there is no *in vivo* evidence supporting a particular mechanism of action for DL-*threo*-β-F-Asn, our previous results using tumor cells in culture show that DL-*threo*-β-F-Asn is incorporated into protein and prevents asparagine-linked glycosylation (11). It is, therefore, possible that a similar effect is occurring *in vivo*. This is supported by observations with tunicamycin, an inhibitor of asparagine-linked glycosylation. Tunicamycin is cytotoxic to tumor cells in culture (13) and is active against the L1210 tumor in mice (9). Thus, if DL-*threo*-β-F-Asn is inhibiting protein glycosylation in the L1210 cells *in vivo*, then this effect might be the mechanism of the observed antitumor activity. In addition to inhibition of glycosylation, the substitution of DL-*threo*-β-F-Asn for L-Asn in protein could also effect protein conformation and, consequently, biological function, since L-Asn has a high probability of being located at β-turns of peptide chains (1).

On the other hand, we do not know whether DL-*threo*-β-F-Asn is metabolized to other cytotoxic agents. A likely metabolite would be DL-*threo*-β-F-Asp. Although the data reported here show that exogenous DL-*threo*-β-F-Asp is not toxic to L1210 cells, our previous results have shown that uptake of DL-*threo*-β-F-Asp is a major determinant of its toxicity (14). Thus, DL-*threo*-β-F-Asn toxicity could result from its intracellular hydrolysis to DL-*threo*-β-F-Asp in L1210 cells.

The results we have obtained showing that DL-*threo*-β-F-Asn is active against L1210 cells in culture are in agreement with those of Duschinsky *et al.* (3), who synthesized DL-*threo*-β-F-Asn via a different route, starting from 5-fluoro-6 dimethoxymethyluracil. This result offers strong evidence that the antileukemia activity of DL-*threo*-β-F-Asn is in fact due to DL-*threo*-β-F-Asn and not to some undetected impurity. DL-*erythro*-β-F-Asn has been synthesized from tert-butyl-β-fluorooxaloacetate (17) and has been found to be inactive *in vivo* against the L-5178Y lymphatic leukemia in CDF mice. Our present studies have shown that the *threo*-diastereomer of DL-β-F-Asn is about 10 times more active in culture than is the *erythro*-diastereomer against the 3 human cell lines, as well as against the L1210 cells. Our previous studies using cells in culture have shown that DL-*erythro*-β-F-Asn is not incorporated into proteins and does not inhibit asparagine-linked glycosylation (6, 11).

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