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USE OF 4-FLUORO-L-ORNITHINE TO MONITOR METABOLIC FLUX THROUGH THE POLYAMINE BIOSYNTHETIC PATHWAY

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Abstract—The mechanistic effectiveness of various polyamine analogs and enzyme inhibitors is typically determined by their ability to deplete intracellular polyamine pools. In this study, we describe an assay that may prove useful in augmenting this relatively static assessment of drug action. The assay relies upon the substitution of 4-fluoro-L-ornithine (Fl-Orn) for ornithine as a polyamine precursor to provide a means to measure metabolic flux through polyamine pools. At concentrations up to 500 μM, the analog did not inhibit the growth of L1210 murine leukemia cells during incubations of up to 72 hr. Using HPLC, the analog was processed metabolically over time to what was deduced to be 2-fluoroputrescine, 6-fluorospermidine and 6-fluorospermine. The relative proportion of fluorinated polyamine analog to the natural polyamine increased with time and Fl-Orn concentration. The sum of the two was found to be nearly identical to the respective polyamine pool of control cells exposed instead to 500 μM ornithine. This indicates that Fl-Orn was recognized and utilized as a precursor at a rate very similar to that of ornithine itself. Using L1210 cells at different stages of cell growth, it was determined that the metabolic flux through the pools, as indicated by the rate of appearance of individual fluorinated polyamine species, reflected the proliferation status of the cells—non-growing cells failed to incorporate the analog. Likewise, in cell types with varying polyamine pool profiles, such as polyamine enzyme overproducers or those with constitutively different spermidine of spermine ratios, the incorporation of the fluorinated analogs into pools was found to be proportional to the size to the natural polyamine pool. In cells treated with inhibitors of *S*-adenosylmethionine decarboxylase, Fl-Orn incorporation indicated a total blockade of polyamine synthesis at that enzyme site. Overall, the Fl-Orn assay has demonstrated that polyamine pool profiles generally reflect the rate of flux through the pathway in proliferating cells, suggesting that most intracellular polyamines are freely exchangeable with those undergoing metabolic flux.

Key words: analogs; 4-fluoro-L-ornithine; metabolic flux; polyamines; polyamine biosynthesis; putrescine; spermidine; spermine

Polyamines and polyamine biosynthetic activity are known to be critical components of the mitogenic response and sustained cell proliferation [1, 2]. Recognizing this fact, polyamine analogs and specific polyamine biosynthetic enzyme inhibitors have been used in antiproliferative strategies to target polyamine biosynthesis, homeostasis, and function [3–6]. The relative success of these strategies is indicated by the recent entry of two polyamine analogs, DENSPM§ [7, 8] and *N,N'*-bis-[3-(ethylamino)-propyl]-1,7-heptane diamine [9], into Phase I clinical trials against solid tumors. It is expected that the polyamine enzyme inhibitor CGP-48664 [10, 11] and another polyamine analog BE-4444 [12], will also enter clinical trial when preclinical drug development is completed.

Typically, the mechanistic effectiveness of such inhibitors and analogs is determined by their ability to affect various polyamine enzyme activities and, more importantly, to deplete and/or perturb intracellular polyamine pools [3–6], as detected by HPLC. These latter determinations are of limited usefulness, however, since they fail to reveal whether the detectable polyamine pools are static or cycling. Thus, while providing an indication of the relative polyamine content of cells, pool size determinations fail to reveal the rate at which Put is converted to Spd and Spd to Spm. In other words, pool size does not necessarily reflect metabolic flux through these pools. Since the half-life of polyamine pools is expected to be no longer than the doubling time of the cell type, it is likely that pool turnover time is much shorter in rapidly proliferating cells—a property that cannot be evaluated by standard HPLC polyamine pool determinations. Moreover, certain polyamine antagonists, such as DENSPM, produce a massive induction in Spd/Spm N^1-acetyltransferase (SSAT) activity [13, 14], which undoubtedly contributes to polyamine pool dynamics [13, 15] by enhancing polyamine excretion and catabolism.

Although metabolic flux can be measured by radioactivity contained in HPLC fractions of cells prelabeled with the tritiated polyamine precursor Orn, the methodology is cumbersome and difficult to apply routinely. Measurement of extracellular levels of the polyamine

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§ Abbreviations: AMA, *S*-(5'-deoxy-5'adenosyl)methylthioethylhydroxylamine; CHO, Chinese hamster ovary cells; CHO/664, Chinese hamster ovary cells that overproduce *S*-adenosylmethionine decarboxylase; L1210/D10, L1210 cells that overproduce ornithine decarboxylase; CGP-48664, 4-amidinoindan-1-one-2'-amidinohydrazone; DFMO, α-difluoromethylornithine; Fl-Orn, 4-fluoro-L-ornithine; Fl-Put, 2-fluoroputrescine; Fl-Spd, 6-fluorospermidine; Fl-Spm, 6-fluorospermine; ODC, ornithine decarboxylase; Orn, ornithine; Put, putrescine; AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; BE-4444, 1,19-bis-(ethylamine)-5,10,15-triazanonedecane; and DENSPM, N^1,N^{11}-diethylnorspermine, also known as N^1,N^{11}-bis(ethyl)norspermine.

by-product, 5′-methylthioadenosine, which is produced stoichiometrically during Spd and Spm synthesis, has been used effectively by at least one group [16] as an evaluation of the polyamine biosynthetic rate. This approach, however, can only be applied with cells that are deficient in 5′-methylthioadenosine phosphorylase and presumably excrete all of the metabolite into the medium to avoid toxicity [17]. Unfortunately, it requires the extrapolation of medium to intracellular biosynthetic events and gives little or no indication of the consequences to individual polyamine pools.

Some time ago, Seiler and colleagues noted that 2,2-difluoroputrescine could be transformed metabolically by both normal [18] and tumor [19] tissues into the spermidine and spermine analogs, 6,6-difluorospermidine and 6,6-difluorospermine, respectively (see Fig. 1). With appropriate HPLC methodology, these analogs could be distinguished from the natural polyamines. In a subsequent study [20], they reported that the monofluoro derivative Fl-Put was transformed more rapidly and efficiently into the higher polyamine analogs than was 2,2-difluoroputrescine. It was observed in Fl-Put-treated animals that the natural polyamine pools accumulated to the highest degree in rapidly proliferating tissues such as tumors and small intestine, suggesting that they may have potential as diagnostic tumor markers [20]. On the basis of these findings, we considered that fluorinated analogs such as Fl-Put might provide a means for following metabolic flux through polyamine pools, since they are distinguishable from natural polyamines by HPLC. Their potential usefulness, however, was limited by the fact that Fl-Put analogs by pass ODC, which together with AdoMetDC is rate-determining for polyamine biosynthesis [21]. It seemed possible that an appropriately fluorinated analog of ornithine could yield Fl-Put after being decarboxylated by ODC. Thus, the analog Fl-Orn (see Fig. 1) was designed and synthesized for evaluation as a marker of polyamine metabolic flux.

In this report, we characterized the biological effects of Fl-Orn on cells and demonstrated that the analog can be readily used and simply detected in a standard HPLC assay to estimate metabolic flux through polyamine pools. Three applications were used to test this possibility: (a) cell lines that overproduce ODC and AdoMetDC, (b) cells treated with AdoMetDC inhibitors, and (c) melanoma cell lines with varying Spd/Spm ratios. Overall, our findings indicated that standard polyamine pool determinations reflect the rate of metabolic flux.

MATERIALS AND METHODS

Materials

L1210 murine leukemia cells were obtained from the American Type Culture Collection (Rockville, MD). DFMO, a specific inhibitor of ODC [22], was provided by Marion Merrell Dow (Cincinnati, OH); the specific inhibitors of AdoMetDC, CGP-48664 [10, 11] and AMA [23] were provided by Ciba Geigy (Basel, Switzerland) and by Drs. Alex and Radii Khomutov (Moscow, Russia), respectively. Fl-Put was obtained originally from Dr. G. A. Digenis (University of Kentucky, Lexington, KY) and later from Dr. N. Seiler (currently, University of Rennes, France).

Fl-Orn

A small quantity of Fl-Orn (Fig. 1) was provided initially as a gift by Dr. Janos Kollonitsch (Merck & Co., Rahway, NJ). Once the potential usefulness of Fl-Orn was demonstrated, the analog was synthesized according to a published procedure (Merck & Co., Inc. US Patent 4004996, Dec. 23, 1974) as follows. In a 1-L two-necked Teflon reactor equipped with a Teflon inlet tube, magnetic stirring bar, Teflon-coated quartz window and UV lamp, L-ornithine hydrochloride (33.7 g, 0.2 mol) was dissolved at −50° in 200 mL of liquid hydrogen fluoride. The solvent was evaporated at room temperature, and the residual L-ornithine hydrofluoride was redissolved in 600 mL of liquid hydrogen fluoride at −57°. The solution was saturated with boron trifluoride (*ca.* 50 g), and a mixture of fluorine: nitrogen (1:10; total 0.2 mol of fluorine) was bubbled into the reaction mixture while stirring and irradiating the mixture with UV light during 20 hr at −78°. The solvent was distilled off and the residue was dissolved in 400 ml of water. To the solution was added 100 g of sodium carbonate in portions (until pH 9.5), followed by a solution of 110 g di-*tert*-butyldicarbonate in 200 mL of tetrahydrofuran. After stirring for 18 hr at 20° the mixture was extracted with ether, and the water layer was treated dropwise at 5°, until pH 2.5, with 4 N hydrochloric acid. The mixture was extracted

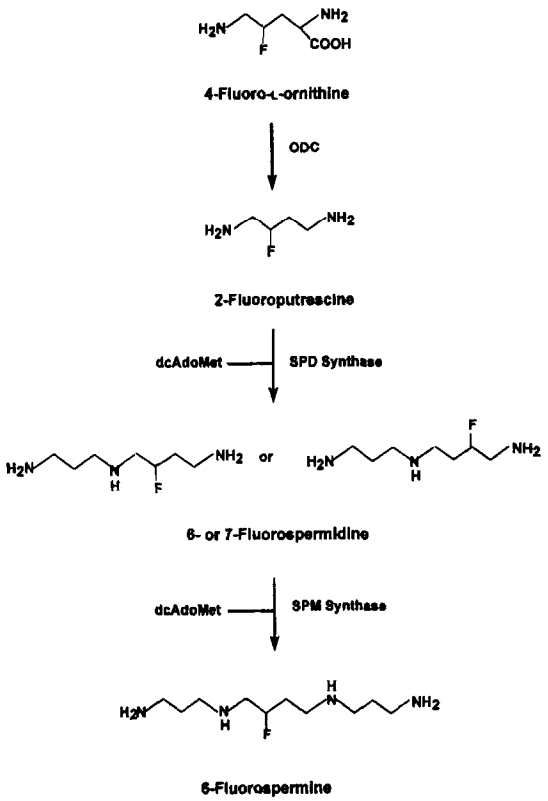

Fig. 1. Biosynthesis of intracellular Fl-Orn. Structure of 4-fluoro-L-ornithine (Fl-Orn) and potential metabolic conversion of Fl-Orn to 2-fluoroputrescine (Fl-Put) via ornithine decarboxylase; the conversion of Fl-Put to 6- or 7-fluorospermidine (Fl-Spd) depending on which terminal amine group of Fl-Put is aminopropylated during the spermidine synthase reaction; and the conversion of Fl-Spd to 6-Fl-Spm. In tissues treated with Fl-Put, Dezeure *et al.* [20] were unable to detect 7-Fl-Spd; therefore, we presume that only the 6-Fl-Spd is synthesized in these cell lines treated with Fl-Orn. dcAdoMet = decarboxylated AdoMet.

immediately with ethyl acetate, and the organic layer was dried over magnesium sulfate and evaporated in vacuum to give crude N,N^1-di-BOC-4-fluoro-L-ornithine hydrochloride as a brown oil (26.9 g).

Part of this oil (10 g, 0.03 mol) was dissolved in 400 mL toluene and treated under reflux with a solution of 6.7 mL (0.045 mol) of *N,N*-dimethylformamide-dimethylacetate in 100 mL of toluene. After the usual work-up, the product was purified by flash chromatography on silica gel with dechloromethane:ethyl acetate (85:15) as the mobile phase to obtain 1.5 g of N,N^1-di-BOC-4-fluoro-L-ornithine methyl ester, m.p. 90–98°, $[\alpha]_D$ = +17.2° (c = 1.074 in $CHCl_3$), MS (FAB): $(M + H)^+$ = 365; Anal. for $C_{16}H_{29}FN_2O_6$ (mol. wt. 364.41): calculated, 52.74% C, 8.02% H, 7.69% N, 5.21% F; found, 53.4% C, 8.0% H, 7.6% N, 5.2% F.

The above-mentioned N,N^1-di-BOC-4-fluoro-L-ornithine methyl ester (3.73 g, 10.2 mmol) was stirred with 40 mL of concentrated hydrochloric acid for 18 hr at 60°. The resulting solution was evaporated to dryness. The residual Fl-Orn dihydrochloride was dissolved in 20 mL methanol and treated with 0.8 mL of pyridine, while 1.5 g of Fl-Orn hydrochloride was obtained, m.p. 210° (decomposition), $[\alpha]_D$ = +4.4° (c = 1.130 in H_2O) MS (FAB): $(M + H)^+$ = 151; ^{1}H-NMR (D_2O): δ = 5.25 and 4.99 (2m, 1H); 3.88 (t, 1H); 3.15–3.45 (m, 2H); 2.1–2.42 (m, 2H). Anal. for $C_5H_{11}FN_2O_2 \cdot HCl$ (mol. wt 186.61):calculated, 32.18% C, 6.48% H, 15.01% N, 19.00% Cl, 10.18% F; found, 32.10% C, 6.32% H, 14.93% N, 19.09% Cl, 10.71% F.

Cell culture

L1210 murine leukemia cells were grown in RPMI 1640 medium containing the semi-defined serum substitute NuSerum IV (Collaborative Research, Boston, MA) and incubated at 37° in the presence of 5% CO_2. Human melanoma cells obtained from G. Giovanella [24, 25] as well as L1210/D10 cells (unpublished) and CHO/664 cells [21] derived by these laboratories, were also adapted to and maintained in this same medium. The medium was normally Orn-free. Cultures were maintained under exponential growth conditions for all experiments. Cells were treated for various times with concentrations of Fl-Orn ranging up to 750 μM. Control cells were always treated with 500 μM Orn in place of Fl-Orn.

HPLC detection of polyamines and fluoro-analogs

Cell samples were extracted with 0.6 M perchloric acid and centrifuged, and the supernatant extract was assayed for polyamines by HPLC using an adaptation of a previously described procedure [26] based on *o*-phthalaldehyde postcolumn derivatization. Polyamines in a 50-μL sample of each perchloric acid extract were separated by an HPLC system using a 50 mm × 4.6 mm steel column packed with DC-4A cationic exchange resin (Sierra Separations, Inc., Reno, NV). The column was maintained at 70° in a column heater (Waters Associates, Milford, MA). The mobile phase consisted of buffer A (0.2 M boric acid, 0.5 M NaCl, 0.03% Brij 35, and 0.0001% octanoic acid, pH 6.0) and buffer B (0.2 M boric acid, 3.1 M NaCl, and 0.0001% octanoic acid, pH 6.0) at flow rate of 1.2 mL/min. A low pressure gradient generator (model M112-3CIM, Autochrome, Milford, MA) and a Waters 590 programmable pump (Waters Associates) were used to mix buffers A and B according to the following step gradient: at 0 min, 100% B; at 10 min, 43% A and 57% B; at 20 min, 67% A and 33% B; at 30 min, 100% A; and at 40 min, reequilibration with 100% B. The column eluate was derivatized at 0.5 mL/min with 0.05% *o*-pthaladehyde (Pierce Chemical Co., Rockford, IL) in 0.4 M borate buffer (pH 10.4) containing 1 mM 2-mercaptoethanol and 0.09% Brij 35 (Pierce Chemical Co.), and then passed through a flow cell of an FL-750 fluorometer (McPherson, Acton, MA) with fixed excitation and emission wavelengths of 360 and 540 nm, respectively. Elution times were as follows: Fl-Put, 18.8 min; Put, 20.2 min; 6- or 7-Fl-Spd, 27.0 min; Spd, 29.3 min; 6-Fl-Spm, 35.5 min; and Spm, 36.9 min. Data were analyzed using a Hewlett Packard HP-9816 computer system and Nelson Analytical chromatography software. With a sensitivity in the range of 20 pmol, polyamine levels were expressed as nanomoles per 10^6 cells. Authentic standards of the natural polyamines and Fl-Put were analyzed separately to identify and quantitate each peak.

Medium preparation for HPLC analysis

To 6 mL of medium, 1.5 mL of 50% trichloroacetic acid was added. The samples were placed on ice for 15 min, and spun at 15,000 *g* for 15 min. The supernatants were collected, 12 mL of 0.01 M ammonium phosphate buffer (pH 8) was added, and the pH was readjusted to pH 8 prior to column extraction. Each solution was loaded onto a Bond Elut carboxylic acid solid phase preparatory column (Analytichem International, Harbor City, CA), washed with buffer, and eluted with 1 mL of 0.1 HCl in methanol. Each sample was dried under nitrogen at 37° and reconstituted with 150 μL distilled water, then 80 μL was loaded onto the HPLC column.

RESULTS

HPLC analog detection with time

Cells were treated with 500 μM Fl-Orn for 8, 24, and 48 hr in the absence of Orn and then assayed for polyamine and fluoropolyamine content by HPLC. In a typical chromatogram (Fig. 2), it was apparent that Fl-Orn was readily metabolized to what appeared to be Fl-Put, Fl-Spd and Fl-Spm (see Fig. 1). With time, the amount of all three fluoro-analogs increased in cells while that of the natural polyamines decreased. The analogs appeared as peaks that migrated just ahead of each of the natural polyamines. The identity of Fl-Put was confirmed by spiking samples with authentic analog and observing the coincident enhancement of the putative Fl-Put peak. The identity of the Spd and Spm analogs was deduced by a comparison of results by Seiler and so-workers [20] in which 2-Fl-Put was shown to be converted to 6-Fl-Spd and 6-Fl-Spm in biological samples. Although this does not represent a definitive identification of the various peaks, it is difficult to envision an alternative interpretation since the precursor, Fl-Orn, differs from Orn by only a single atom.

Concentration dependence

L1210 cells were treated with concentrations of Fl-Orn ranging from 100 to 750 μM for 24 hr (Fig. 3). Cellular extracts were found to contain fluoro-analogs at all Fl-Orn concentrations. Although there was a slight increase in the proportion of analogs to natural polyamines as Fl-Orn increased from 100 to 500 μM, the

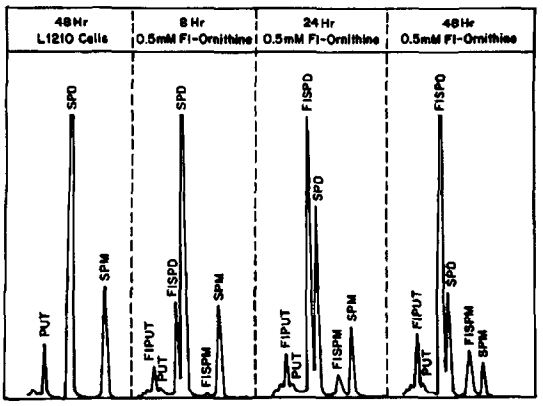

Fig. 2. Representative HPLC chromatogram of natural and fluoro-derived polyamine pools. L1210 cells incubated in 500 μM Fl-Orn for various times (8–48 hr) were analyzed by HPLC. Note that the fluorinated polyamine analogs appear as separate peaks from the natural polyamines and that their permeation into the polyamine pools increases with time. These chromatograms were generated from cells processed under steady-state growth conditions and were typical of those taken from three experiments. There was only one Fl-Spd peak, which in theory could represent 6-fluorospermidine and 7-fluorospermidine (see Fig. 1). Attempts were not made to separate the two since it has been shown previously [20] that only 6-fluorospermidine is biologically generated from Fl-Put.

relative increase at 750 μM was minimal. Thus, it appeared that the system was nearly saturated at 500 μM. On the basis of this information, 500 μM Fl-Orn was selected for all subsequent studies. As described below (Fig. 4), this concentration was not growth inhibitory to L1210 cells during incubations for up to 48 hr. Relative to Orn-treated cells, growth inhibition averaged 4% for 100 μM Fl-Orn, 5% for 250 μM Fl-Orn, 7% for 500 μM Fl-Orn, and 10% for 750 μM Fl-Orn.

Effects on cell growth

By concentration–response evaluation, the growth of L1210 cells was affected minimally by the presence of Fl-Orn at concentrations up to 1000 μM and for up to 72 hr (Fig. 4A). However, when treatment was extended (i.e. 72 hr following reseeding at 48 hr), growth was affected in a concentration-dependent manner. Substitution of 500 μM Orn for Fl-Orn during this same time period had no effect on cell growth (data not shown). In an attempt to determine whether growth inhibition was due to Fl-Orn effects on polyamine metabolism or Orn metabolism, cells were also treated with Fl-Put (Fig. 4B). For a relative comparison, cells treated for 24 hr with 100 μM Fl-Put achieved fluoro-analog pools equivalent to those treated with 500 μM Fl-Orn (data not shown). At 250 μM, Fl-Put was only slightly growth inhibitory under extended treatment conditions.

Time dependence

L1210 cells were treated with 500 μM Fl-Orn for up to 48 hr—a period equivalent to four cell generations. As shown in Fig. 5, the relative proportion of Fl-Orn incorporated into the total Put pool remained constant during this time, whereas that incorporated into Spd and Spm pools increased steadily. At 8 hr, there was detectable fluoro-analog found in each of the pools. By 48 hr, Fl-Spd comprised about 75% of the total Spd pool and Fl-Spm about 70% of the total Spm pool. It should be noted that, as with the studies on concentration dependence, the combined analog plus natural polyamine for any given pool was almost identical to natural polyamine pool size for cells treated with an equivalent concentration (500 μM) of Orn (Figs. 3 and 5). Growth inhibition averaged 0% for the 8-hr treatment with 500 μM Fl-Orn, 7% for 24 hr, and 16% for 48 hr. Although Fl-Orn treatment for as little as 12 hr would be sufficient to achieve pool permeation in L1210 cells, 24 hr was selected for subsequent L1210 cell studies.

Time dependence and growth rate

Analog permeation of polyamine pools was compared in L1210 cells at different phases of cell growth. Cells were allowed to grow for 48, 72, or 96 hr during which time growth decreased steadily. Twenty-four hours prior to harvesting, cells were pulse-labeled with 500 μM Fl-Orn as opposed to the previous experiment (Fig. 5) where cells were treated continuously with Fl-Orn. As can be seen from Fig. 6A, the L1210 cells initially grew rapidly with an average of two cell doublings per 24-hr period. Beyond 72 hr, their growth rate fell sharply. During rapid growth at both 48 and 72 hr, Fl-Orn readily permeated all three polyamine pools (Fig. 6B). After 48 hr Put and Spd polyamine pool sizes began to decrease, and after 72 hr incorporation of Fl-Orn into Spd and Spm pools ceased. Once cells ceased dividing (i.e. after 72 hr), there was no apparent permeation of the pools by the analog, indicating that the pools were essentially static.

Enzyme overproducers

The effect of atypical polyamine metabolism was also examined. Fl-Orn incorporation into the polyamine pools was studied in L1210 cells, which overexpress ODC (L1210/D10), and in CHO cells, which overexpress AdoMetDC (CHO/664). Parental cells and enzyme overproducing cells were grown out of drug selection for 96 hr and incubated for 24 hr in medium containing 500 μM Fl-Orn or Orn. This incubation period allowed permeation of all three pools, as evidence by the appearance of Fl-Spm. The polyamine and polyamine pool data are presented in Fig. 7. L1210/D10 cells expressed 10-fold higher ODC activity than parental cells and accumulated a massive steady-state pool of Put. Consistent with this profile, large amounts of Fl-Put were observed in the L1210/D10 cells, while less Fl-Spd and Fl-Spm, were seen relative to the parental L1210 cells. The findings suggest that the unusually large Put pool in the L1210/D10 cells is (a) not compartmentalized, (b) freely exchanges with Fl-Put, and (c) serves to dilute the Fl-Put available for use in subsequent synthesis. Overall, the pattern of Fl-Orn incorporation tends to follow the profile of the natural polyamine pools themselves.

CHO/664 cells stably overproduce AdoMetDC. Removal of the selecting agent, the AdoMetDC inhibitor CGP-48664, leads to a rapid conversion of both Put and Spd to Spm, leaving the former pools depleted and that of Spm increased by a factor of ~2 [21]. In these cells, polyamine pools are the opposite of those seen in ODC-overproducing cells. Since there was essentially no Put or Spd pools in the CHO/664 cells, Fl-Spm accumulated to more than twice that accumulated in parental cells, again mimicking the profile of the natural polyamine pools.

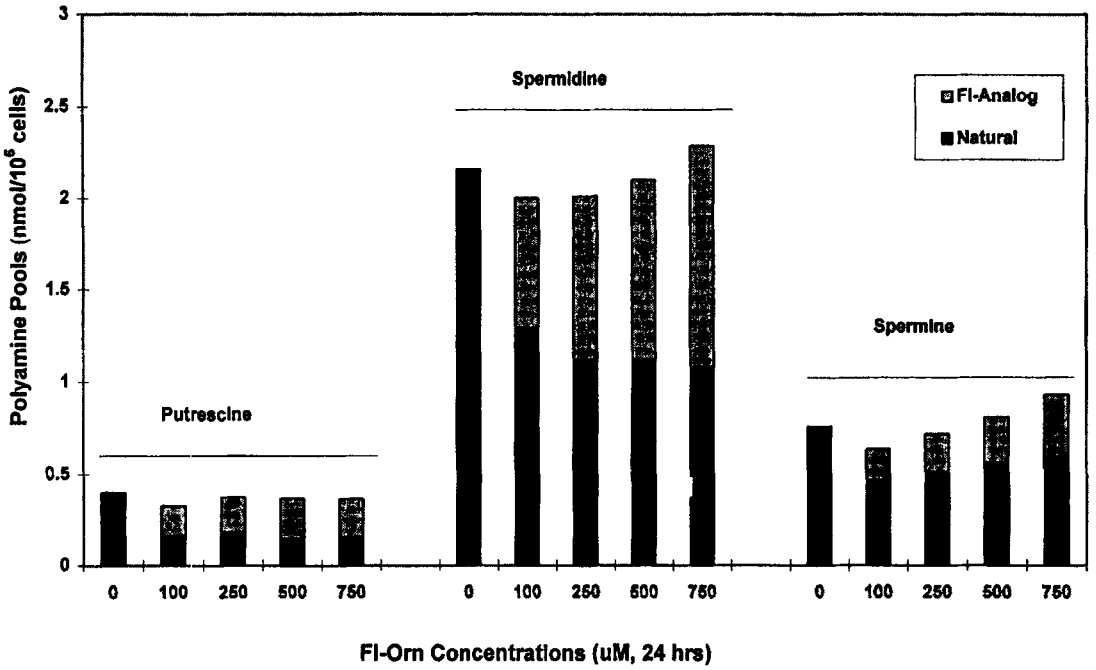

Fig. 3. Concentration-dependent incorporation of fluoro-analogs into L1210 cell polyamine pools: Polyamine and polyamine analog content of L1210 cells incubated with increasing concentrations of Fl-Orn for 24 hr. Untreated cells were incubated with 500 μM Orn. Each column depicts the natural polyamine (dark pattern) and the Fl-analog (light pattern) content of cells, determined by HPLC analysis. The data represent the means of two experiments performed in duplicate. Standard deviations were all less than 17%.

AdoMetDC inhibition

To gain better insight into polyamine pool dynamics and metabolic responses to inhibition of polyamine biosynthesis, Fl-Orn incorporation was examined in L1210 cells treated with the AdoMetDC inhibitors AMA [23] and CGP-48664 [11]. This experiment was performed at concentrations that produced ~50% growth inhibition at 48 hr, 100 and 4 μM, respectively. As shown in Fig. 8, approximately 50% of the total Put pool accumulated during treatment with either inhibitor was Fl-Put, indicating that there was continued flux into this pool. Only trace amounts of Fl-Spd and Fl-Spm were observed in the drug-treated cells where Spd and Spm pools were essentially depleted. The lack of Fl-Orn permeation beyond Fl-Put indicated that the block by either inhibitor was virtually complete. As found previously, the sums of Fl-Spd plus Spd and Fl-Spm plus Spm in the Fl-Orn treated cells were very similar to the Spd and Spm pools of cells treated with Orn alone (data not shown). There were, however, definite differences in the Put pools (Table 1). Whereas Put in cells treated with CGP-48664 or CGP-48664 plus Orn rose to ~10.0 nmol/10^6 cells, the combined Fl-Put plus Put pools in cells treated with the inhibitor plus Fl-Orn were found to be less than half that amount (4.73 nmol/10^6 cells). Upon further examination, it was determined that although Fl-Put was synthesized to the same extent as Put in the presence of this AdoMetDC inhibitor, a large proportion of the Put analog (8.7 nmol/10^6 cells) was excreted into the medium instead of being retained intracellularly. Thus, when the total Put and Fl-Put pools found in the cells and the medium were compared following Fl-Orn and Orn treatment, they were similar (14.0 and 14.7 nmol/10^6 cells, respectively). It should be noted that growth inhibition by either CGP-48664 or AMA was unexpectedly 10–20% greater in cells treated concomitantly with Fl-Orn.

Metabolic flux in cells with different Spd/Spm pool ratios

We next examined the permeation of Fl-Orn into the Spd and Spm pools of seven human melanoma cell lines previously found to have constitutively different steady-state Spd/Spm pool ratios (Table 2). Cell cultures were treated with 500 μM Orn (control) or Fl-Orn for 48 hr. The longer incubation period was required in these cells to ensure permeation to the final metabolite, Fl-Spm. Polyamine pool analysis revealed that Fl-Orn-derived analogs distributed among the pools in a manner consistent with the Spd/Spm ratio of each cell type. Thus, the natural Spd/Spm pool ratios for all cell types were very similar to those for Fl-Spd/Fl-Spm, and were nearly identical to the ratio of total (natural + fluoro-polyamine) Spd/Spm pools. Since the Spm content of each cell type was comparable, the Spd/Spm ratios of 0.20, 0.21, 0.31, 0.46, 0.67, 0.83, and 0.98 were determined by the Spd content. Consistent with this fact, the Fl-Spd content in Fl-Orn-treated cells also varied, whereas the Fl-Spm pools were comparable. This is somewhat surprising since the amount of Fl-Spd available for conversion to Fl-Spm was much higher in LOX, Ebey, MALME-3M, and HO melanoma cells.

DISCUSSION

Several findings reported here support the tenet that Fl-Orn can be used simply and effectively to evaluate

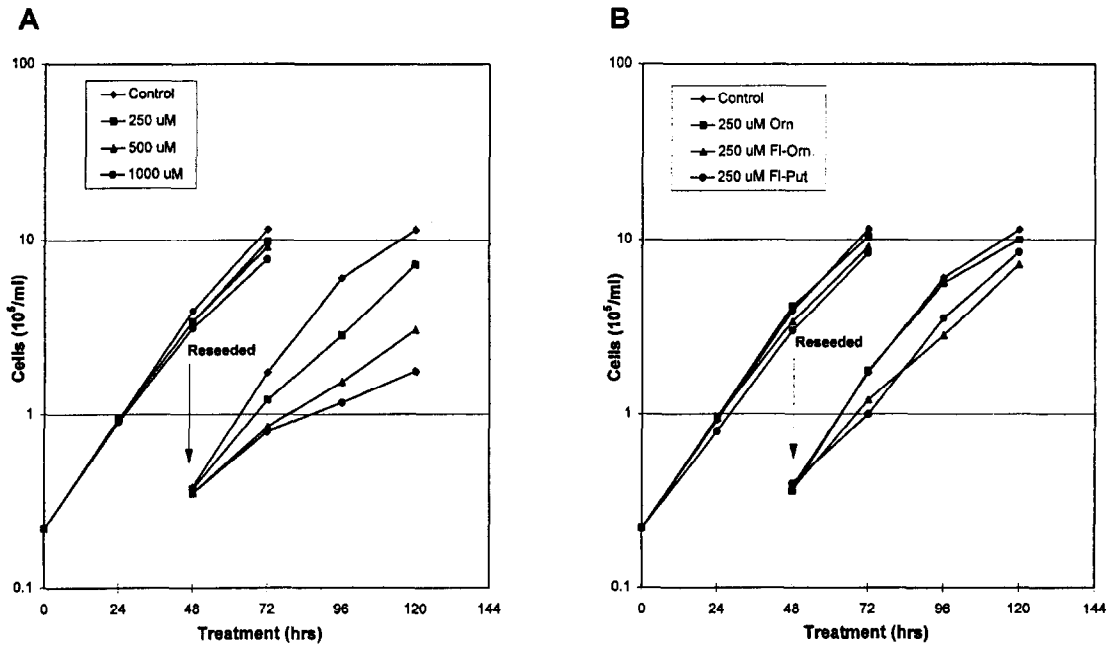

Fig. 4. Effect of Fl-Orn, Orn and Fl-Put on cell growth. Shown are growth curves of L1210 cells treated with various concentrations of Fl-Orn (A, left panel) for 120 hr with reseeding and retreating at 48 hr. For comparison purposes, cells were treated similarly with Fl-Orn, Orn or Fl-Put at 250 μM, the maximum concentration possible with available Fl-Put (B, right panel). Data represent the averages of three experiments with standard deviations <15%.

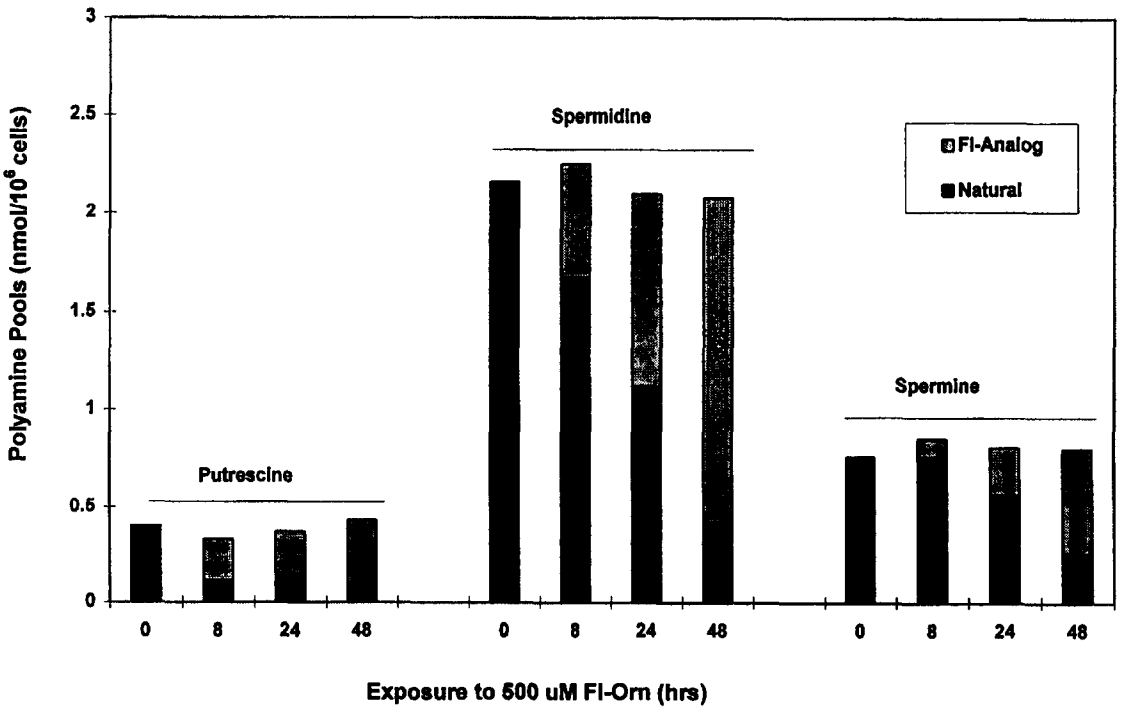

Fig. 5. Time-dependent incorporation of Fl-Orn into L1210 cell polyamine pools (continuous exposure): Polyamine and polyamine analog content of L1210 cells incubated for 0–48 hr in 500 μM Fl-Orn. Untreated cells (shown at 0 time) were incubated in 500 μM ornithine in place of Fl-Orn. Each column depicts the natural polyamine (dark pattern) and the Fl-analog (light pattern) content of cells. These data represent the means of two experiments performed in duplicate. Standard deviations (not shown) were all less than 13%.

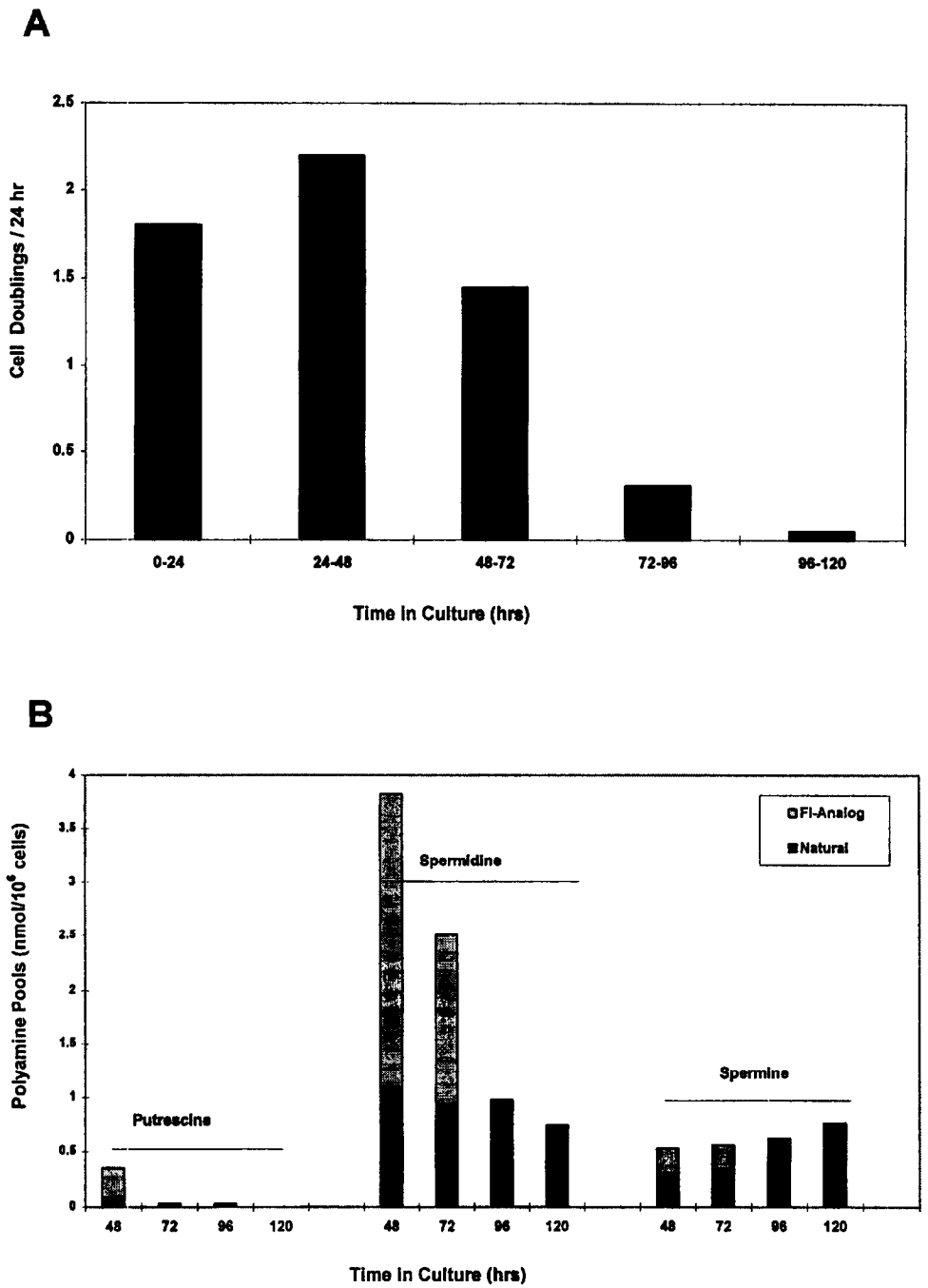

Fig. 6. Time-dependent incorporation of Fl-Orn into L1210 cell polyamine pools (pulse exposure). (A) L1210 cell-doubling times during time in culture. Cells were seeded at 0.3×10^5/mL and sampled for cell counting at 24, 48, 72, 96 and 120 hr. (B) Incorporation of Fl-Orn into the intracellular polyamine pools in L1210 cells during different stages of cell growth. Cells were incubated for 24 hr in 500 μM Fl-Orn prior to harvesting at 48, 72, 96 and 120 hr. These data represent the averages of duplicate samples at each time point.

metabolic flux through intracelllular polyamine pools. First, we have established that Fl-Orn is well-tolerated by cells at concentrations up to 1 mM during exposures up to 72 hr. Second, the Fl-Orn derived fluorinated polyamine analogs are readily detectable and quantifiable by the straightforward HPLC methodology described here. Although the identities of the Fl-Spd and Fl-Spm peaks were not confirmed with authentic standards, they are consistent with earlier findings by Dezeure *et al.* [20] in cells incubated with Fl-Put. Finally, we have demonstrated that the Fl-Orn is metabolically processed through to the highest polyamine, Fl-Spm (Fig. 1) with the same apparent efficiency as the natural polyamine precursor Orn. In support of this fact, we note that it was found repeatedly among different cell types and under different treatment conditions that the sum total of the

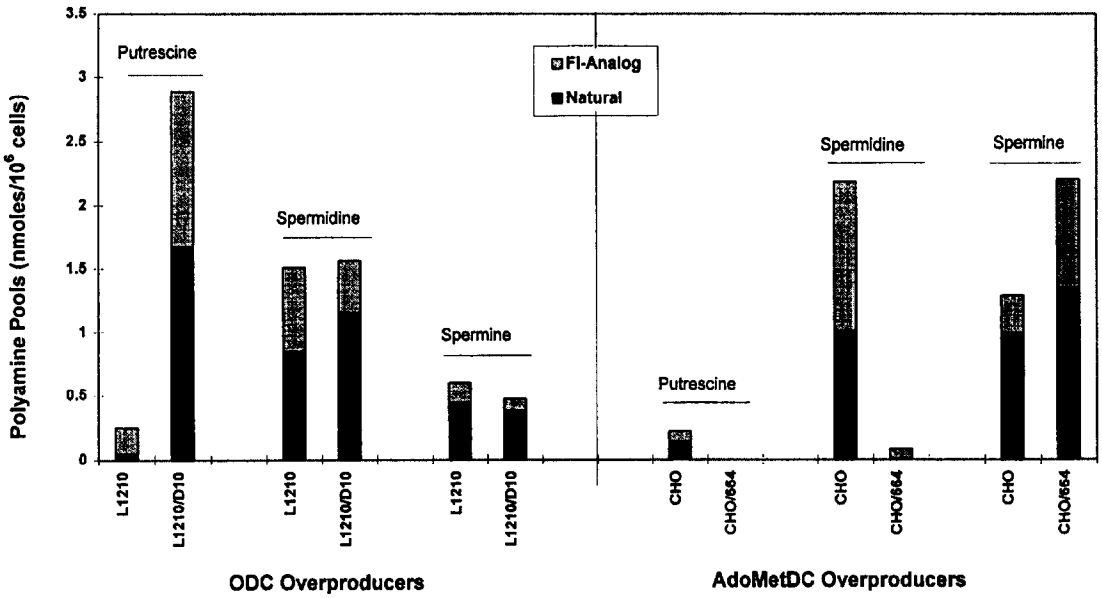

Fig. 7. Fl-Orn incorporation into cells that overproduce ODC or AdoMetDC. Shown is the Orn-derived analog permeation of polyamine pools in ODC overproducing L1210 cells (L1210/D10, left panel) and AdoMetDC overproducing CHO cells (CHO/664, right panel) treated for 24 hr with 500 μM Fl-Orn. Each column depicts the natural polyamine (dark pattern) and the Fl-analog (light pattern) content of cells. These data represent the averages of three separate determinations with standard deviations <10%.

fluoro-labeled polyamines and the natural polyamines consistently matched the individual polyamine pools of Orn-treated cells. Thus, not only is Fl-Orn utilized similarly to Orn, but the subsequently derived polyamine analogs, Fl-Put, Fl-Spd and Fl-Spm, are metabolized at the same rate as the natural polyamines. This is consistent with the earlier report by Dezeure *et al.* [20] who found that Fl-Put is a good substrate for Spd synthase

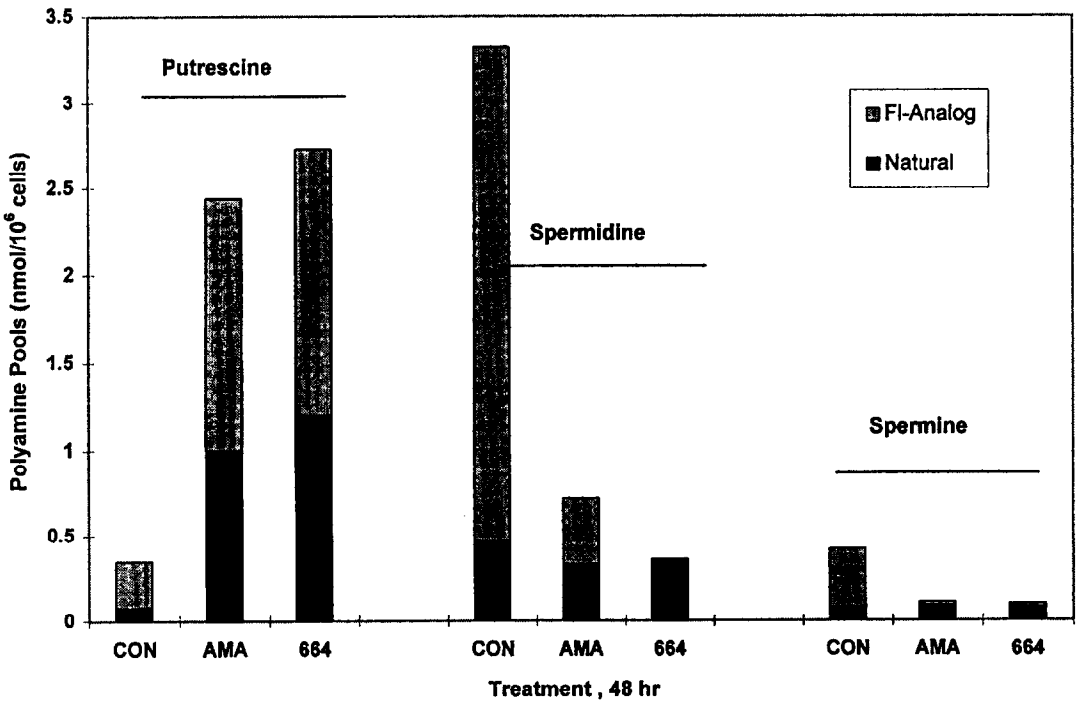

Fig. 8. Effects of AdoMetDC inhibitors on Fl-Orn permeation of polyamine pools. L1210 cells were treated for 48 hr with 100 μM AMA or 4 μM CGP-48664 (664) plus 500 μM Fl-Orn during the entire treatment period. Each column depicts the natural polyamine (dark pattern) and the Fl-analog (light pattern) content of cells. These data represent the means of three experiments with standard deviations less than 10%.

Table 1. Synthesis of Put and Fl-Put in the presence of an AdoMetDC inhibitor

Treatment (48 hr)	Intracellular*		Medium†		Total Put + Fl-Put (nmol/10^6 cells)
	Fl-Put	Put	Fl-Put	Put	
	(nmol/10^6 cells)				
Control L1210	0	0.39	0	0.76	1.15
4 μM CGP-48664	0	9.42	0	2.48	11.9
4 μM CGP-48664 plus 500 μM Fl-Orn	2.23	2.5	8.71	0.58	14.0
4 μM CGP-48664 plus 500 μM Orn	0	10.33	0	4.39	14.7

* These data represent the averages of three separate determinations with standard deviations <10%.
† Units, nmol found in the equivalent mL for 10^6 cells; the milliliters for each sample varied as a consequence of growth inhibition.

and that, like Fl-Orn, it is readily converted to Fl-Spd and Fl-Spm. In a further evaluation of this finding, Seiler's group showed that Fl-Put is an effective substrate for spermidine synthase [27] and that it is capable of substituting for Put in activating the enzyme AdoMetDC [28].

The metabolic flux assay was evaluated in several different ways. In the first, L1210 cells were treated with Fl-Orn for periods up to 48 hr in order to demonstrate the existence of metabolic flux under growth conditions. As per expectations, Fl-Orn moieties permeated the polyamine pools in increasing proportions to Fl-Spd and Fl-Spm with time so that by 48 hr, the latter comprised ~70% of the total Spm pool. As noted above, the combined natural and analog pools of the individual polyamines always resembled the natural polyamine pool of Orn-treated cells, suggesting that Fl-Orn is utilized at a rate comparable to the natural precursor Orn. In addition, the relative proportion of one polyamine pool to another (for example, the Put, Spd, and Spm pool profile of a particular cell type) in Fl-Orn-treated cells always approximated that of the natural polyamine pool profile in Orn-treated (control) cells, further confirming the biological similarity of the analog to Orn.

Since it is presumed that cell division utilizes intracellular polyamine pools, rapidly dividing cells would be expected to incorporate more Fl-Orn than more slowly dividing cells. Our findings with dividing and nondividing L1210 cells support this concept. In particular, pulse-labeled L1210 cells that were dividing incorporated Fl-Orn moieties into all three polyamine pools, whereas nondividing (confluent) cells failed to incorporate any Fl-Orn. Of the various polyamine perturbations studied here, nondividing cells represent the only instance in which Fl-Orn incorporation failed to reflect the general profile of the intracellular polyamine pools. It suggests that the pools of non-dividing L1210 cells are metabolically static. The finding supports the long-standing therapeutic premise underlying the preclinical development of polyamine antagonists as potential anticancer agents, namely that non-dividing cells should be relatively unaffected by polyamine inhibitors largely because the biosynthetic enzymes are down-regulated. However, since other cell types may have a greater potential to excrete and/or metabolize polyamines when not dividing, this conclusion may not represent a generality.

In L1210 cell ODC-overproducers, considerable Fl-Orn was found in the large Put pool and slightly less in

Table 2. Fluoro-analog permeation of polyamine pools in human melanoma cell lines with constitutively different steady-state polyamine pool ratios

Cell line	Treatment (48 hr)	Polyamine pools*				Ratios†		
		Fl-SPD	SPD	Fl-SPM	SPM	SPD/ SPM	Fl-SPD/ Fl-SPM	Total SPD/ Total SPM
		(nmol/10^6 cells)						
PaNut melanoma	Control	0	0.53	0	2.62	0.20	NA‡	
	500 μM Fl-Orn	0.32	0.39	1.37	1.90	0.21	0.23	0.22
SH1 melanoma	Control	0	0.58	0	2.78	0.21	NA	
	500 μM Fl-Orn	0.53	0.52	1.45	1.96	0.26	0.38	0.31
STO melanoma	Control	0	0.67	0	2.20	0.31	NA	
	500 μM Fl-Orn	0.47	0.60	0.98	1.64	0.36	0.47	0.39
LOX melanoma	Control	0	1.11	0	2.42	0.46	NA	
	500 μM Fl-Orn	0.70	1.08	1.74	1.96	0.55	0.40	0.48
Ebey melanoma	Control	0	1.97	0	2.92	0.67	NA	
	500 μM Fl-Orn	0.62	1.53	0.97	2.33	0.66	0.64	0.65
MALME-3M	Control	0	1.95	0	2.36	0.83	NA	
	500 μM Fl-Orn	0.73	1.22	0.73	1.68	0.73	1.00	0.80
HO melanoma	Control	0	2.86	0	2.91	0.98	NA	
	500 μM Fl-Orn	1.38	1.14	1.32	1.45	0.79	1.04	0.91

* Means of at least four separate determinations with standard deviations for the Fl-Orn-treated cells within 15% of each mean value.
† Total ratio; combination of natural polyamine and/or fluoro-analog. Compare with ratio of control natural polyamine pool.
‡ NA, does not apply.

the Spd and Spm pools relative to parental cells, presumably because the Fl-Put has to compete with the relatively large remaining pool of Put for conversion into Spd and Spm and their analogs. In the case of the AdoMetDC-overproducing CHO cells, the opposite occurred with the Fl-Orn moieties apparently moving quickly through the Put and Spd pools to Spm. These data indicate that in cells with enzyme activities augmented by stable gene amplification, the increased rate of flux is also reflected by the Fl-Orn metabolites.

With both of the above cell types, the Fl-Orn-derived polyamine analogs distributed in the polyamine pools in proportion to the total polyamine pools themselves. This same tendency was also noted among human melanoma cells that constitutively contain different Spd/Spm ratios where it was again observed that the Fl-Orn moieties assumed a relative distribution among the pools which reflected that of the total polyamines themselves. Thus, cells with a high total natural Spd/Spm ratio display a similarly high Fl-Spd/Fl-Spm ratio. The observation suggests that polyamine sequestration or compartmentalization does *not* occur even in cells such as the ODC overproducers that contains an unusually large putrescine pool. Likewise, the argument could be made that although a certain proportion of polyamines are undoubtedly bound in the cell, the present data suggest that they either exchange freely with unbound polyamines or that the proportion of bound polyamines is similar for all three polyamines.

When cells are treated with various polyamine inhibitors, polyamine pools are perturbed in specific ways often leading to the accumulation of specific polyamine species. At issue is the degree to which metabolic flux occurs in such accumulated pools. In the case of the ODC inhibitor DFMO, Spm tends to accumulate. However, 500 μM Orn or Fl-Orn prevented growth inhibition in cells treated with 1 mM DFMO (data not shown), presumably by competing with this inhibitor for intracellular uptake and/or at the active site of ODC. Due to the difficulties in interpretation of such experiments, these conditions were not studied further. In the case of AdoMetDC inhibitors such as CGP-48664 and AMA, putrescine pools accumulate due to a compensatory rise in ODC and loss of AdoMetDC activities, while Spd and Spm pools decrease. We used Fl-Orn to gain insight into polyamine pool dynamics during typical responses to these inhibitors. Findings indicate that the biosynthetic blockade by either inhibitor is relatively complete. This allows metabolic flux into the Put pools, as indicated by the accumulation of Fl-Put during treatment, but almost none into either Spd or Spm pools. Since it has been suggested that Put is able to substitute for Spd in some growth-related functions during treatment with AdoMetDC inhibitors [23], an interesting distinction was noted between Put and Fl-Put in this regard. When Put conversion to the higher polyamines was blocked by AdoMetDC inhibition, most (~80%) of the accumulated Put pool remained in the cells; however, in the case of Fl-Put, most (~80%) was excreted into the medium. This suggests that the cells attempt to avoid toxicity due to Fl-Put accumulation. Furthermore, unlike Put, Fl-Put is apparently unable to support cell growth.

Overall, the data presented here indicate that Fl-Orn incorporation can be used as a rapid and simple means to measure the rate of metabolic flux through intracellular polyamine pools. Future studies will examine how regulatory effects on polyamine enzymes, such as c-*myc* transactivation of ODC [29] or inhibitor-mediated depletion of AdoMet pools [30], impact on flux through the polyamine pools.

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