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LIGAND BINDING AND KINETICS OF FOLATE RECEPTOR RECYCLING IN VIVO: IMPACT ON RECEPTOR-MEDIATED DRUG DELIVERY

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Running Title: Ligand binding and kinetics of folate receptor recycling

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Abstract

Folate receptor-targeted cancer therapies constitute a promising treatment for the approximately 1/3 of human cancers that over-express the folate receptor (FR). However, the potencies of all folate-receptor targeted therapies depend on i) the rate of folate-linked drug conjugate binding to the cancer cell surface, ii) the dose of folate conjugate that will saturate tumor cell surface FR *in vivo*, iii) the rate of FR internalization, unloading, and recycling back to the tumor cell surface for another round of conjugate uptake, iv) and the residence time of the folate conjugate before its metabolism or release from the cell. Because little information exists on any of these processes, we have undertaken to characterize them on both cancer cells in culture and solid tumors in live mice. We quantitate here the properties of FR saturation, internalization, recycling, and unloading in several cultured cancer cell lines and murine tumor models, and we describe the conditions that should maximize both the potencies and specificities of folate receptor-targeted therapies *in vivo*.

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Introduction

The folate receptor (FR) is a tumor marker that is over-expressed on a variety of human cancers, including cancers of the ovary, kidney, lung, breast, brain, endometrium, and myeloid cells of hematopoietic origin (Leamon et al., 1993; Lu and Low, 2002; Reddy and Low, 1998, 2000; Wang and Low, 1998). Because folic acid is internalized by FR-mediated endocytosis, it has been exploited as a delivery agent for the transport of attached molecules that don't ordinarily enter cancer cells. Importantly, conjugation of molecules to folic acid does not normally interfere with folate's high affinity for its receptor nor with its endocytosis into the cell (Turek et al., 1993; Leamon and Low, 1991, 1992 and 1993). Thus, linkage of a drug to folic acid can generate a molecular "Trojan Horse" that can enable tumor-specific delivery of both imaging and therapeutic agents into cancer cells. Molecules that have been targeted to tumors by conjugation to folic acid include radiopharmaceuticals, (Wang et al., 1996, 1997; Mathias et al., 1996, 1998; Leamon et al., 2002). enzymes for pro-drug activation (Lu et al., 1999), nanoparticles (Oyewumi and Mumper; 2003), peptides, toxic proteins (Leamon and Low; 1992), immunologically potent haptens (Lu and Low; 2002), antisense oligonucleotides (Citro et al., 1994; Wang et al., 1995), chemotherapeutic agents (Ladino et al., 1997), gene therapy vectors (Leamon et al., 2001; Reddy et al., 1999, 2002; Lee and Huang, 1996), viruses (Reddy and Low, 2000; Lee and Low, 1994), polymeric drug carriers and liposomes (Lee and Low, 1995; Gabizon et al., 1999). Importantly, both a folate-targeted therapeutic drug and a folate-linked imaging agent are currently under evaluation in human clinical trials.

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The pathway by which the occupied folate receptor is internalized into a cancer cell has been a matter of much debate. Some research has pointed to internalization of FR at caveolae (Rijnboutt et al., 1996; Ritter et al., 1995; Smart et al., 1994; Chang et. al., 1992; Rothberg et al., 1990) while other studies have established that FR are endocytosed at clathrin-coated pits (Rodman et al., 1986, Birn et al., 1993). Maxfield and colleagues have cautioned that the trafficking pathways taken by a recycling receptor such as FR can be complex and may involve many sorting events in multiple organelles (Maxfield and McGraw; 2004). Regardless of their internalization mechanism, knowledge of the rates of receptor binding, endocytosis, trafficking to an organelle for ligand release, and recycling back to the cell surface can greatly assist in defining the concentration and frequency of drug dosing for optimal efficacy. Thus, administration of folate-drug conjugates more frequently than the rate of reappearance of empty FR at a tumor cell surface should simply increase toxicity without improving potency, while dosing drug conjugates at concentrations insufficient to saturate cell surface FR will under-utilize the capacity of the tumor-specific delivery system.

Motivated by these considerations, we have endeavored to quantitate both the conditions for FR saturation and the frequency of FR recycling in a variety of cancer cells and animal tumor models using folate-linked radioimaging agents. We report that significant differences exist in FR recycling rates among various cancer cell lines, as well as between cancer cells and kidney proximal tubule cells. We also observe that while different tumor types have different FR contents, they saturate *in vivo* at similar folate conjugate concentrations. From these data, we provide fundamental information that should be useful in defining the optimal concentration, administration frequency,

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and type of folate-drug conjugate for the imaging and treatment of FR-expressing cancers.

Materials and Methods

Cell lines and culture. A panel of cancer cells, HeLa, KB, HS578T, MDA231, and Line 01, expressing different levels of FR/mg cell protein were obtained from the Purdue Cancer Center or Endocyte, Inc. (West Lafayette, IN). M109 cells were obtained from Alberto Gabizon (Hadassah-Hebrew University Medical School), and L1210A cells, selected for high FR expression by continuous culture in low folate medium, were a gift from Gerrit Jansen (University Hospital, Amsterdam, The Netherlands), and IGROV cells were a gift from Patrick Hwu (The National Cancer Instititute, Bethesda, MD). All cell lines were cultured at 37^{o}C in a humidified atmosphere containing 5% CO_2. The cells were grown continuously either as a monolayer or in suspension in folate deficient Dulbecco's Modified Eagle's medium (FDMEM) (Gibco BRL, Gaihersburg, MD) supplemented with 10% heat inactivated fetal bovine serum (Hyclone Labrotories, Logan UT), penicillin (50 units/ml), streptomycin (50 ug/ml) and 2 mM L-glutamine. The normal complement of endogenous folates in the fetal bovine serum brings the net folate concentration in the growth medium into the low end of the physiological range of folate concentrations found in human plasma. Dialyzed fetal bovine serum supplemented with 1 μM folinic acid was used for culturing L1210A cells.

Tumor models. Five to six week old Balb/c and DBA-2 mice were obtained from Harlan (Indianapolis, IN) or Purdue University Breeding Colony (West Lafayette, IN). All mice were maintained on a folate deficient diet (DYETS, Inc. Bethlehem, PA) for the duration

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of tumor growth in order to allow their serum folate levels to decrease into the physiological range (Mathias et al., 1996). M109, L1210A, and Line 01 tumors were generated by subcutaneous injections of $1*10^6$ M109, $5*10^5$ L1210A and $1*10^6$ Line 01 cells, respectively, on the dorsal side of the mouse. Tumors were then allowed to grow to ~50mm^2 before initiation of the receptor saturation and recycling studies described below.

Preparation of folate-DTPA-In^{111}. Folate-DTPA-^{111}In was prepared by chelating non-carrier-added ^{111}indium chloride (Mallinckrodt Medical. Inc., St Louis, MO) with folate-DTPA (diethylenetriaminepentaacetic acid-ethylenediamine-γ-folate) (a generous gift from Endocyte, Inc) (Wang et al., 1997, Mathias CJ and Green MA, 1998). For this purpose, a dilute acidic solution of $^{111}In^{3+}$ was initially complexed with sodium citrate and then incubated for 3h at room temperature with an aqueous solution of folate-DTPA to allow transfer of the radionuclide from the citrate to the more stable folate complex. The radiochemical purity of folate-DTPA-^{111}In was found to exceed 92% by analysis on Whatman paper or C_{18} reverse phase thin layer chromatography using methanol as a mobile phase. Folate-DTPA-^{111}In was mixed with cold folate-DTPA-In to obtain the concentration and specific activity needed for evaluating the kinetics of folate receptor endocytosis. Because the half life of the radioactive indium is 67.23 h, extended studies of the trafficking of the folate conjugate in tumor-bearing mice were possible.

Evaluation of the kinetics of folate-FITC uptake into KB cells by confocal microscopy. Confocal microscopy was used to document the rapid binding and endocytosis of folate

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conjugates by a representative cancer cell line (KB cells, derived from a human nasopharyngeal cancer). For this purpose, 1 x 10^4 KB cells in 0.5ml of growth media were deposited into glass bottom Petri dishes and incubated overnight at 37^{o}C to encourage strong adherence and optimal subconfluent cell spreading. Spent incubation medium was then replaced with 0.5 mL of fresh serum-supplemented medium containing 50 nM folate-FITC, and the cells were incubated at 37ºC for 0, 5, 10, 30, 60, and 120 min. After rinsing 4x with 0.5 mL of Hanks-buffered saline containing 10 mg/ml unlabeled bovine serum albumin, cell-associated folate-FITC was imaged using a confocal laser scanning microscope.

Measurement of internalized and membrane-bound folate-H^3/folate-DTPA-In^{111} following various periods of continuous incubation. To quantitate the amount of cell surface and internalized folate-^{3}H or folate-DTPA-^{111}In, cells were plated in 24-well plates at approximately 40% confluence and allowed to grow 48 h before each experiment. Either folate-^{3}H or folate-DTPA-In^{111} was then added to the cultured cells, and the cells were incubated for the times indicated in the figures. Cells were then washed with PBS and stripped to remove unbound folate-DTPA-^{111}In or folate-^{3}H by washing twice with 0.5 M NaCl, 10 mM Na-acetate, adjusted to pH 3.5. Cells were then lysed in 0.5 ml of 1% Triton X-100 in PBS. The number of molecules endocytosed/cell was determined from the protein content and radioactivity in each solution, and the rate of conjugate internalization was estimated from the change in the above value over the 6h incubation time.

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Analysis of saturation of folate receptor with folate-DTPA-In^{111} in vivo. To determine the concentration of folate-DTPA-^{111}In required to saturate M109, Line 01 and L1210A tumors, and normal tissues, a dose-escalation study was performed using folate-DTPA-^{111}In concentrations of 50, 100, 500, 750, 1000, 1250, 1500, 2000, and 4000 nmoles/kg. Briefly, the different concentrations of folate-DTPA-^{111}In were injected intraperitoneally into the tumor-bear mice and the mice were sacrificed 4 h later by cervical dislocation. For distribution analyses, blood, heart, lung, spleen, liver, kidney, intestine, muscle and tumor were harvested, and the radiation levels in each tissue were determined with a gamma counter (Packard BioScience Co. Meridian, CT).

Analysis of the rate of folate receptor recycling in vivo. The strategy for analysis of the rate of FR recycling *in vivo* was based on the following three premises: 1) that only empty cell surface receptors can mediate endocytosis of newly added folate-DTPA-^{111}In, 2) that de novo synthesized receptors represent only a small fraction of the empty FR that appear at the cell surface, and 3) that empty cell surface receptors arise primarily from the cycling of FR through acidic intracellular compartments, since folate dissociation from extracellular FR at neutral pH is very slow (Kamen and Smith, 2004). Therefore, following initial administration of a saturating dose of folate-DTPA-^{111}In (determined using the methods described above), the rate of appearance of empty FR on the cancer cell surface can be taken as an estimate of the rate of FR recycling through its intracellular compartments. To measure this rate of appearance of empty FR on the cancer cell surface, an initial saturating dose of folate-DTPA-^{111}In is administered.

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Then, at various time points, a second saturating dose of the same conjugate is again injected. If additional tumor uptake of folate-DTPA-^{111}In is not observed, then insufficient time must have elapsed for FR to release its cargo inside the cell and return to the cell surface. In contrast, if measurable additional uptake is observed, then intracellular unloading and cell surface recycling of empty FR must have transpired. Thus, by noting the time interval before additional folate-DTPA-^{111}In can be taken up by the tumor, the minimum time for FR recycling can be estimated. For this purpose, six different frequencies of injection of a saturating concentration of folate-DTPA-^{111}In were administered over a 40 h period. All mice were then sacrificed at 48 h, and their tumors/normal tissues were weighed and counted. The frequencies and times of injection were 1X at 40 h; 2X at 0 and 40 h; 3X at 0, 20 and 40 h; 4X at 0, 13.5, 27 and 40 h; 6X at 0, 8, 16, 24, 32 and 40 h; and 8X at 0, 5.5, 11, 16.5, 22, 28, 33.5 and 40 h. Finally, all tumor-bearing mice were sacrificed 8 h after the last injection, thereby allowing an equal amount of time for clearance of unbound and nonspecifically bound folate-DTPA-^{111}In from the tissues. . Tissues examined were blood, heart, lung, spleen, liver, kidney, intestine, muscle and tumor.

Evaluation of Folate Conjugate Retention Following Endocytosis In Vivo. To determine the rates of folate-DTPA-^{111}In release from M109, L1210A, and Line 01 tumors, as well as normal tissues in vivo, a saturating dose of ^{111}In-DTPA-folate (~2,500 nmole/kg) was injected intraperitoneally (i.p.) at time zero and the mice were sacrificed (by cervical dislocation) at times 4, 8, 24, 48, 72 and 120 h post injection. Again, for these biodistribution analyses, blood, heart, lung, spleen, liver, kidney, intestine, muscle and

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tumor were harvested, and radiation in each tissue was determined by counting in a gamma counter (Packard BioScience Co. Meridian, CT). The first order kinetics of conjugate release were quantitated by plotting the log of tissue radioactivity versus time.

Results

Kinetics of receptor-mediated endocytosis of folate conjugates by a panel of FR-positive cancer cells. Six cancer cell lines, expressing different levels of FR/cell, were analyzed for their abilities to bind and internalize three different folate-targeted reporter molecules: 1) folate-FITC, 2) ^{3}H- folate, and 3) folate-DTPA-^{111}In. For this purpose, each of the folate derivatives was added to the indicated cells in culture, and both the total cell-associated radioactivity/fluorescence as well as the fraction of conjugate that had been internalized (see Methods) were evaluated as a function of time. Importantly, since co-addition of 100 μM unlabeled folic acid as a competitive inhibitor invariably blocked >99% of the binding/uptake of each compound (data not shown), and because the reduced folate carrier does not transport folate conjugates (Leamon et al., 2002), internalization of the above compounds was assumed to be catalyzed by FR-mediated endocytosis. As seen by both confocal microscopy and scintigraphic analysis (Fig. 1), all 3 folate conjugates associate rapidly with the cells in culture and approach receptor saturation within the initial 30 min of incubation. Continued exposure of the cells to the folate-linked reporter molecules then results in sustained, but considerably slower uptake that persists for at least the 6h duration of the study (Fig. 1B). Thus, although total receptor-mediated binding varies from 3 to 18 x10^{6} folate-DTPA-^{111}In and ^{3}H- folate per cell (Fig. 1B), the rapid phase of binding is always complete within 30 min, and the rate

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of conjugate internalization, as measured by the residual cell-associated radioactivity after acid stripping, gradually increases at a rate of ~1-3 x 10^5 molecules/h for all cells except the MDA231 cells; see Figs. 1B and 2A). Thus, although the HeLa, KB, IGROV, M109, and HS579T cancer cell lines differ in FR expression by at least a factor of six, they all internalize folate-drug conjugates at a relatively similar rate that does not appear to correlate with the folate binding capacity of the cell. Further, since no major differences are observed between the amounts of ^{3}H- folate and folate-DTPA-^{111}In internalized, it can be concluded that folate conjugates are endocytosed as efficiently as free folic acid. This latter observation is consistent with other studies showing that the affinity of folic acid for its receptor is not usually compromised by its attachment to other molecules (Leamon et al., 2002). Further, since ^{3}H- folate can also enter cells slowly through the reduced folate carrier, whereas folate-DTPA-In^{111} cannot, the similar rates of uptake between the two reporter molecules also implies that internalization of oxidized folate by these cells is primarily mediated by FR. Finally, it is interesting to note that by the 6 h incubation point, only ~10-25% of the total cell-associated radioactivity had been internalized in any of the cell lines (Fig. 2B). These data suggest that most of the folate conjugates either remain on the cell surface or recycle through the cell interior without unloading, as previously suggested by others (Kamen and Capdevila, 1986).

Dose escalation study of folate-DTPA-In^{111} in vivo. Dose escalation studies were next performed to determine the concentration of folate-DTPA-^{111}In that would saturate L1210A, M109, and Line 01 tumors, as well as normal tissues *in vivo*. Thus, although

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the binding affinity of folate-DTPA-^{111}In for FR in cultured cells is well established (K_d~1x10^{-9} M), the dose of folate-conjugate that will saturate cell surface FR in solid tumors *in vivo* has been difficult to predict, primarily because this saturating dose is heavily influenced by the rates of i) solid tumor perfusion and receptor binding, ii) folate-DTPA-^{111}In excretion, and iii) folate-DTPA-^{111}In metabolism by other tissues. Therefore, to determine the concentration of folate conjugate that must be administered to achieve tumor saturation *in vivo*, a dose escalation study using folate-DTPA-^{111}In as a model ligand was conducted. For this purpose, mice bearing L1210A, M109, and Line 01 tumors were injected intraperitoneally with increasing concentrations of folate-DTPA-In^{111} ranging from 50-4,000 nmole/kg, and after allowing 4h for clearance of unbound conjugates from the tissues, tumor and normal tissues were dissected, weighed, and counted. As shown in Fig 3, L1210A tumors, M109 tumors, Line 01 tumors, and kidneys all saturated at folate-DTPA-^{111}In concentrations of ~2,000 ±300 nmole/kg. In contrast, other normal tissues accumulated negligible quantities of the conjugate (data not shown). Furthermore, when tumor-bearing mice were administered folate-DTPA-^{111}In at the above saturating dose, net accumulation of the conjugate in L1210A tumors (~250 nmole/kg) significantly exceeded net uptake by the kidney (~100 nmole/kg), which in turn was higher than uptake by M109 (~50 nmole/kg) or Line 01 (~20 nmole/kg) tumors, as shown in Fig. 3. Importantly, the highest tumor to blood contrast (as estimated by the ratios of percent injected dose per gram tumor and blood) ranged from 328 to 500 in Line 01, M109, and L1210A tumors from mice administered 100, 500, and 750 nmole/kg of folate-DTPA-In^{111}, respectively, and these concentrations also corresponded to the doses where the optimal tumor to liver and muscle contrast were

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similarly obtained (Table 1). Optimal tumor to kidney contrast, however, was not seen until ~1000-2000 nmole/kg conjugate.

Rate of folate receptor recycling in vivo. The recycling rate of the folate receptor was characterized in L1210A, M109, and Line 01 tumors as well as normal tissues by saturating the tissues with a single dose of folate-DTPA-^{111}In, allowing the complexes to endocytose, and measuring the delay before empty receptors could reappear on the tumor cell surfaces (see Methods). Experimentally, this delay was estimated by administering saturating doses of folate-DTPA-^{111}In at increasingly shorter time intervals and determining when further reduction in the time interval no longer led to an increase in net tumor or normal tissue uptake (i.e. when total accumulation in the tissues began to plateau). At this dosing interval, the frequency of FR recycling should correspond to the frequency of folate-DTPA-In^{111} administration. For the present study, dosing intervals of 40h, 20h, 13.3h, 8h and 5.7h were examined, and an 8h washout period was included at the end of each experiment to ensure that unbound folate conjugates would have time to clear from the tissues before counting. Based on these measurements, the rate of FR recycling *in vivo* was estimated to be slightly less than 5.7 hours for L1210A cells, but near 13.3 to 20 hours for Line 01 and M109 cells (Fig. 4A). Recycling of FR in the kidney occurred at a frequency much greater than every 5.7 hours, as also indicated in Fig. 4A. Although the heart, lung, liver, spleen, intestine, and muscle expressed considerably fewer FR than the tumor cells, they still recycled their receptors with frequencies near ~8h (Fig. 4B). Interestingly, the tumor to background contrast was generally observed to decrease at increasing injection frequencies for M109

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and Line 01 tumors, but not for L1210A tumors, as shown in Table 2. We ascribe these trends to the differences in FR recycling rates between the different tumors and normal tissues. Thus, where FR recycling in the tumor is distinctly slower than in the tissues, as observed for M109 and Line 01 tumors, increasing the dosing frequency would be expected to favor uptake by the tissues. In contrast, where FR recycling rates in tumor and normal tissues are similar, no change in tumor to normal tissue ratio with dosing frequency would be expected.

A very different situation obtains when the dose of folate conjugate is small compared to the dose required for tumor saturation. Under these conditions, higher dosing frequencies invariably show better tumor to normal tissue ratios (Fig. 4C and D). For example, after 6 injections at 8 h intervals of 100 nmoles/kg folate-DTPA-^{111}In, a tumor to liver ratio of 34 was measured, whereas upon similar dosing at 2000 nmoles/kg, a tumor to liver ratio of only 3 was observed. This increase in tumor to normal tissue ratio with dosing frequency at low folate-DTPA-^{111}In concentrations likely derives from the much higher binding capacity of the tumor than normal tissues and the fact that tumor saturation may not easily be achieved with repeated dosing at these low concentrations.

Rate of release of folate-DTPA-In^{111} in vivo. Another variable that can influence the potency of a folate-targeted drug concerns length of time that the conjugate remains associated with the cancer cell. Thus, if the tumor retention time is long, multiple reactions/processes can occur following uptake, such as release of the drug from the vitamin or redistribution of the drug to the nucleus or some other organelle within the

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cell. In contrast, if retention time is short, the drug's action must be rapid or efficacy may not be fully achieved *in vivo*. To obtain a crude measure of the retention time of a folate conjugate by M109, L1210A, and Line 01 tumors, as well as by normal tissues, tumor-bearing mice were injected with a single saturating dose of folate-DTPA-^{111}In (1,800-2,500 nmole/kg) at time zero, and then sacrificed at times 8, 24, 48, 72, and 120 hours and evaluated for tissue radioactivity. As shown in Fig. 5A, the unloading rates of folate-DTPA-In^{111} were similar for all tumors and the kidney. In fact, the first order rate constants, measured from the log of the net accumulation of conjugate as a function of time, was ~6.2 X 10^{-3} and 5.2 X 10^{-3} nmole/kg/hour, for all three tumors and kidney, respectively. Thus, on average, tumors and kidney release ~12% of their accumulated folate-DTPA-^{111}In by 24 h, 30% by 48 h, 60% by hour 72, and ~80% by hour 120. Further, as shown in Fig. 5B, even though other normal organs (except kidney) do not take up or release significant amounts of folate-DTPA-^{111}In, their rates of unloading of folate-DTPA-^{111}In were still similar to tumor and kidney. Interestingly, the best tumor to normal tissue ratio was seen ~48 hours post-administration of folate-DTPA-In^{111}, as shown in Table 3. Thus, while good contrast with folate-linked imaging agents can be achieved by 2h post injection (Wang et al., 1997), the best contrast should occur ~48h post injection.

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Discussion

We have undertaken to evaluate the physiological variables that can impact the efficacy of folate receptor-targeted therapies *in vivo*. First, the time between folate conjugate injection and receptor binding was considered, since folate-drug constructs that are inactivated during circulation (i.e., via hydrolysis, reduction, oxidation, etc.) will lose potency as time for delivery to the targeted tissue increases. Importantly, binding of three distinct low molecular weight folate derivatives to a panel of FR-expressing cancer cells *in vitro* was found to be complete within 30 min, suggesting FR binding is rapid when permeability barriers are absent. Although similar studies could not be conducted *in vivo* because of the difficulty of distinguishing bound from free folate-linked compounds in the interstitial spaces between tumor cells, data from intravital two-photon fluorescence microscopy revealed that folate conjugates penetrate solid tumors within a few seconds of tail vein injection. (Kennedy and Low, unpublished data) Since the kinetics of folate conjugate binding to accessible FR should not change with the location of the cell, we suggest that uptake of low molecular weight folate conjugates by tumor cells *in vivo* should follow a time course roughly comparable to that measured *in vitro*.

Because folate conjugates that never encounter an unoccupied FR can only contribute to nonspecific toxicity, it became important to determine the folate conjugate concentration where FR saturation occurs *in vivo*. For this purpose, dose escalation studies were conducted on three distinct tumors and several normal tissues. Surprisingly, within experimental error, FR saturation occurred in all tumors and normal tissues at roughly the same concentration, i.e. ~2000 nmol/kg. Because FR saturation in

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cell culture is achieved at two orders of magnitude lower concentration (Leamon et al., 2002), one can conclude that most of the folate conjugates must be excreted or destroyed before they ever encounter an FR-expressing cell *in vivo*. Indeed, preliminary data suggest that the half-lives of several folate conjugates in circulation are <5 min, predominantly as a consequence of their rapid clearance by the kidneys (Leamon et al., 2002). Such short half lives are generally desirable for receptor-targeted therapeutics, since drug constructs that don't encounter a receptor are rapidly removed from the body before they can cause toxicity to nontargeted tissues.

A third variable that can significantly influence the properties of receptor-targeted therapies is the rate of receptor recycling. Data from our FR recycling studies *in vivo* suggest that the rates can vary among different tumors and tissues, but are generally ≤0.05/h. Careful evaluation of this recycling rate is important, since administration of a saturating dose of a folate-drug conjugate more often than the frequency of FR recycling will simply result in maintenance of a high concentration of nontargeted drug, which can only increase the toxicity to all tissues equally. Indeed, although some variability is seen in the data, maximal tumor to normal tissue ratios are generally achieved when a saturating dose is administered at a slightly lower frequency than the frequency of FR recycling in the tumor. Thus, the optimal tumor to liver ratio is seen in L1210A cells (which recycle their FR every ~6 h) at a ~13 h dosing frequency, while the optimal tumor to liver ratio in M109 and Line 01 tumors (which recycle their FR every ~20h) is seen at a ~40 h dosing frequency. However, when significantly subsaturating doses of folate conjugates are administered, optimal tumor targeting can still occur at high dosing frequencies if the binding capacity of the tumor significantly exceeds that of the normal

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tissues, as shown in Fig. 4. Obviously, the choice of conjugate concentration and dosing frequency will depend on whether a continuous or intermittent exposure to the targeted drug is more effective. For our folate-targeted immunotherapy (Lu and Low, 2002), significantly more immune cells are found to enter the tumor when low doses are administered frequently than when high doses are administered infrequently (Wang and Low, manuscript in preparation).

The final variable examined, the rate of release of the targeted drug from the tumor cell, is especially important in the initial design of a tumor-targeted therapeutic agent. Thus, if the tumor were to retain the folate conjugate for only a short period, then tumor cell uptake, release of the unmodified drug from the targeting ligand, binding of the drug to its intracellular target, and disruption of cell function would have to occur within this short time frame. However, since the folate conjugate examined in this study remains tumor cell associated for several days following uptake, ample time exists for all of the aforementioned processes to occur. Based on these and other considerations, we would suggest that linkers connecting folic acid to the drug of choice should be designed to last longer than the 30 min required for folate conjugates to find and bind the tumor cells, yet hydrolyze within the many hours that the conjugate resides within the cancer cell, releasing the unmodified drug to perform its lethal function.

We were surprised to observe that the accumulation of both folate and folate conjugates within the cultured cancer cells was largely independent of the level of FR expression (Fig. 1B). Conceivably, folate/FR trafficking into the cells is driven by a metabolic need for folic acid, and not by a need to regularly cycle all FR between the cell surface and cell interior. If this hypothesis is valid, then a similar number of FR/cell

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may commonly recycle, leaving the remaining population of FR to sit idle on the cancer cell surface. The fraction of folate receptors that recycle is obviously important, since some therapies only require drug delivery to the cancer cell surface (e.g. the folate-targeted immunotherapy) (Lu and Low, 2002) whereas other therapies (e.g., folate-targeted chemotherapy) (Ladino et al., 1997) depend on drug transport into the cell interior.

The similar rates of ^{3}H-folate and folate-DTPA-^{111}In deposition in the cell interior were also not anticipated (see Fig. 2B). Most hypotheses on FR endocytosis/potocytosis suggest that following membrane invagination, folate is released from its receptor (probably due to compartment acidification), and then enters the cytosol by transport through some type of anion transporter (Anderson et al., 1992). Curiously, folate-DTPA-^{111}In is neither a substrate of any known anion transporter nor of the reduced folate carrier. However, since its delivery into the cell interior closely matches that of ^{3}H-folate, escape of the folate/folate conjugates from the endosome may not occur via the postulated anion carrier. Further studies are obviously necessary to identify the nature of this transport/release mechanism.

In summary, folate-targeted therapies show considerable potential for the treatment of cancer and inflammatory diseases (Turk et al., 2002; Lu and Low, 2002; Leamon and Low, 2001; Paulos et al., 2004; Paulos, Varghese, Turk, and Low, manuscripts in preparation and in press) Optimal use of the therapeutic strategy, however, requires a knowledge of i) the time between folate conjugate administration and FR binding, ii) the concentration required for FR saturation *in vivo*, iii) the rate of FR recycling *in vivo*, and iv) the duration of folate conjugate residency in the tumor cell before its escape/release

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into the extracellular milieu. We have provided data that bear on all of these parameters in murine tumor models. Hopefully, they can help guide the selection of an optimal dosing concentration and frequency for those folate conjugates that are ready to advance to the clinic.

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Acknowledgements

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Footnotes

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Legends for figures

Figure 1. The uptake of folate-FITC, folate-H^3, and folate-DTPA-^{111}In as a function of time during continuous incubation for 6 hours in a panel of high FR cancer cells (HeLa, KB, IGROV, HS578T, M109 and MDA231). Figure A. Uptake of folate-FITC as a function of time during continuous incubation from 0 to 2 hours in high FR KB cancer cells, as shown by confocal microscopy. Figure B. Surface bound (□,Δ) and internalized (■,▲) ^{3}H-folic acid (□,■) and folate-DTPA-^{111}In (Δ,▲) in cancer cell lines, as a function of time during continuous incubation with ^{3}H-folate and folate-DTPA-^{111}In. The data represent the mean ± standard error from three time profiles and were plotted using GraphPad Prism.

Figure 2. Rate of internalization of conjugate (Figure A) and percent internalized conjugate (Figure B) in various cell lines after 6 h incubation with folate-H^3 (■) or folate-DTPA-^{111}In (□). The data represent the mean ± standard error from three experiments.

Figure 3. Effect of dose of folate-DTPA-^{111}In on accumulation of the conjugate in tumors and kidney in tumor-bearing mice. Concentration-dependent association of folate-DTPA-^{111}In in FR-expressing L1210A, M109 and Line 01 tumors and kidney sacrificed 4 hours post-administration with increasing doses from 0-4000 nmole/kg of folate-DTPA-^{111}In. The accumulation of folate-DTPA-^{111}In in L1210A tumors is greater

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than the accumulation in the kidneys. In contrast, the accumulation of the conjugate in M109 and Line 01 tumors is less than the accumulation in kidneys. The data represent the mean ± standard error from four dose-profiles and were plotted using GraphPad Prism.

Figure 4. The frequency of FR recycling of folate-DTPA-^{111}In in three different tumor-bearing mice. Uptake of folate-DTPA-^{111}In in FR-expressing tumors, kidneys (Figure 4A) and FR-negative organs (Figure 4B) from L1210A, M109 and Line 01 tumor-bearing Balb/c and DBA mice administered every 5.7 (8 injections/40 hours), 8 (6 injections/40 hours), 13.3 (4 injections/40 hours), 20 (3 injections/ 40 hours) and or 40 hours (2 injections/40 hours) at 1,800-2,500 nmole/kg/injection to determine the recycling of the folate receptor, i.e. the internalization, unloading and trafficking back to the cell surface. Figure 4C. The accumulation of folate-DTPA-^{111}In in M109 tumor-bearing mice administration a saturating dose (2000nmole/kg) compared subsaturating doses (100 nmole/kg) every eight hours at 6 times in 40 hours. Figure 4D. The ratio of accumulation of tumor to tissues in tumor-bearing mice injected 6 times in 40 hours with either 100 or 2000 nmole/kg of conjugate. All tumor-bearing mice were administered a final injection of folate-DTPA-^{111}In at hour 40, and all animals were then sacrificed at hour 48. The data represent the mean ± standard error from three frequency profiles and were plotted using GraphPad Prism.

Figure 5. Residual folate-DTPA-^{111}In in tumors and normal tissue as a function of time. Mice bearing M109, L1210A, and Line 01 tumors were administered a saturating dose

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of folate-DTPA-^{111}In (1,800-2,500 nmole/kg) at time zero and residual conjugate was evaluated in the tumors and tissues over 120 hours. The unloading rate of the conjugate from the tumor and kidney (Figure A) and the normal tissues (Figure B) are similar. As shown in Figure B, nominal amount of the conjugate accumulates in the normal tissue. Thus, on average, tumors and kidney release ~12% of their accumulated folate-DTPA-^{111}In by 24 h, 30% by 48 h, 60% by hour 72, and ~80% by hour 120. The data represent the mean ± standard error from three time profiles and were plotted using GraphPad Prism.

Tables

TABLE 1A

Effect of dose of folate-DTPA-^{111}In on accumulation of the conjugate in the tumor at increasing concentrations of the conjugate.

	Dose of folate-DTPA-^{111}In (nmole/kg)								
Dose	50	100	500	750	1000	1250	1500	2000	4000
Tumor	**Percent injected dose per gram tumor**								
M109	11.8	9.56	6.74	3.76	--	2.98	2.59	1.59	1.23
SEM ±	0.22	1.52	0.67	0.24	--	0.21	0.52	0.24	0.24
Line 01	--	6.55	2.4	1.51	1.27	--	0.83	0.76	0.65
SEM ±	--	1.01	0.78	0.12	0.52	--	0.12	0.08	0.02
L1210A	21.5	--	18.9	16	--	--	--	9	6.25
SEM ±	7.1	--	3.55	1.24	--	--	--	0.62	0.41

Tumor-bearing mice were sacrificed 4 hours post-administration of respective doses of folate-DTPA-^{111}In. Values are presented as means ± S.E.M. of three to four experiments (5 mice per group).

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TABLE 1B

Ratio of dose of folate-DTPA-^{111}In on accumulation of the conjugate in the tumor to normal tissue at increasing concentrations of the conjugate.

	Dose of folate-DTPA-^{111}In (nmole/kg)								
Dose	50	100	500	750	1000	1250	1500	2000	4000
Ratio of percent injected dose per gram tumor to percent injected dose per gram normal tissue.									
M109/Liver	19.3	25.8	39.9	13.4	--	10.6	15.2	22.1	12.7
M109/Kidney	0.25	0.29	0.64	0.54	--	0.85	0.78	0.49	0.55
M109/Muscle	10.7	15.2	23.2	25.1	--	32.7	23.5	14.5	18.1
M109/Blood	194	187	396	376	--	186	173	113	16
Line 01/Liver	--	7.19	3.75	2.32	4.37	--	4.88	4.47	3.43
Line 01/Kidney	--	0.16	0.19	0.24	0.26	--	0.21	0.22	0.15
Line 01/Muscle	--	7.11	24.3	35.9	18.1	--	3.32	3.62	2.65
Line 01/Blood	--	328	120	65.6	17.6	--	26.8	24.5	21.3
L1210A/Liver	19.5	--	16.8	34.5	--	--	--	24.7	17.8
L1210A/Kidney	0.48	--	1.67	2.12	--	--	--	2.78	2
L1210A/Muscle	12.5	--	18.1	62.3	--	--	--	12.9	12.5
L1210A/Blood	53.6	--	146.6	500	--	--	--	315	284

Tumor-bearing mice were sacrificed 4 hours post-administration of respective doses of folate-DTPA-^{111}In. Values are representative of three experiments (5 mice per group).

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TABLE 2A

Accumulation of folate-DTPA-^{111}In in tumors as a function of injection frequency.

		Number of Injections in 40 hours					
# of injections		**1**	**2**	**3**	**4**	**6**	**8**
Injection times (hr)		40	0,40	0, 20,40	0,13.3, 26.6, 40	0,8,16 24,32,40	0,5.7,11.4 17.1,22.8, 8.5,34.2,40
		Percent injected dose per gram tumor					
M109		3.98	2.61	1.76	1.49	0.985	0.712
	SEM	± 0.74	0.62	0.32	0.12	0.09	0.002
Line 01		1.72	1.14	0.89	0.75	0.642	0.257
	SEM	± 0.22	0.29	0.09	0.13	0.12	0.08
L1210A		3.22	5.64	4.03	3.63	3.08	2.62
	SEM	± 0.78	0.96	0.82	0.44	0.34	0.27

Tumor-bearing mice were injected at different time intervals with a saturating dose of folate-DTPA-^{111}In, and all mice were administered the conjugate at 40 hours. Values are presented as means ± S.E.M of three to four experiments (5 mice per group).

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TABLE 2B

Ratio of the accumulation of folate-DTPA-In[111] in tumors to tissue as a function of injection frequency.

	Number of Injections in 40 hours					
# of injections	**1**	**2**	**3**	**4**	**6**	**8**
Injection times (hr)	40	0,40	0,20,40	0,13.3, 26.6, 40	0,8,16 24,32,40	0,5.7,11.4, 17.1 22.8,28.5, 34.2, 40
Ratio of percent injected dose per gram tumor to percent injected dose per gram normal tissue						
M109/Liver	11.4	11.9	7.33	5.52	2.97	3.96
M109/Kidney	0.739	0.5	0.349	0.321	0.272	0.211
M109/Muscle	12.3	20.1	12.6	5.96	5.76	10.6
M109/Blood	151	130	121	62.6	31.6	17.8
Line 01/Liver	15.6	14.9	11.4	8.92	5.33	1.86
Line 01/Kidney	0.5	0.57	0.39	0.33	0.27	0.12
Line 01/Muscle	5.05	37.8	20.7	13.1	14.8	3.29
Line 01/Blood	123	127	148	68.2	53.3	6.9
L1210A/Liver	7.44	5.75	4.15	11.7	7.89	1.94
L1210A/Kidney	1.32	1.63	1.15	1.26	1.12	1.2
L1210A/Muscle	16.7	10.4	11.1	20.5	20.5	29.1
L1210A/Blood	380	156	111	90.7	66.9	52.4

Tumor-bearing mice were injected at different time intervals with a saturating dose of folate-DTPA-^{111}In, and all mice were administered the conjugate at 40 hours. Values are representative of three experiments (5 mice per group).

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TABLE 3A

Residual folate-DTPA-^{111}In in tumor as a function of time post-administration of conjugate (2,000 nmole/kg) at hour 0.

Tumor		Time of Sacrifice (hrs) 8	24	48	72	120
		Percent injected dose per gram tumor				
M109		3.28	2.84	1.73	1.08	0.4
	SEM	± 0.29	0.31	0.21	0.09	0.07
Line 01		1.72	1.51	1.33	0.69	0.32
	SEM	± 0.32	0.45	0.35	0.13	0.07
L1210A		8.25	7.5	6.76	3.85	2.64
	SEM	± 1.48	1.22	0.74	0.32	0.64

Tumor-bearing mice were injected with 2000 nmole/kg of folate-DTPA-^{111}In at time 0 , and sacrificed at 8, 24, 48, 72, and 120 h. Values are presented as means ± S.E.M of three to four experiments (5 mice per group).

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TABLE 3B

Ratio of the accumulation of folate-DTPA-In^{111} in tumors to tissue as a function of injection frequency.

	Time of Sacrifice (hrs)				
	8	**24**	**48**	**72**	**120**
Ratio of percent injected dose per gram tumor to percent injected dose per gram normal tissue					
M109/Liver	16.1	13.1	14.4	10.6	6.95
M109/Kidney	0.63	0.58	0.611	0.521	0.6
M109/Muscle	10.3	13.1	13.8	7.74	4.75
M109/Blood	64.5	92	127	111	52.7
Line 01/Liver	10.8	10.8	13.7	10.2	3.9
Line 01/Kidney	0.41	0.41	0.489	0.312	0.213
Line 01/Muscle	24.6	37.8	48.7	21.5	5.33
Line 01/Blood	71.1	75.5	133	98.6	40
L1210A/Liver	66	55	45.1	19.2	11.6
L1210A/Kidney	2.61	2.51	2.81	1.96	1.61
L1210A/Muscle	22.3	19.7	45.1	24.1	26.4
L1210A/Blood	206	375	606	392	106

Tumor-bearing mice were injected at different time intervals with a saturating dose of folate-DTPA-^{111}In, and all mice were administered the conjugate at 40 hours. Values are representative of three experiments (5 mice per group).

Figure 1

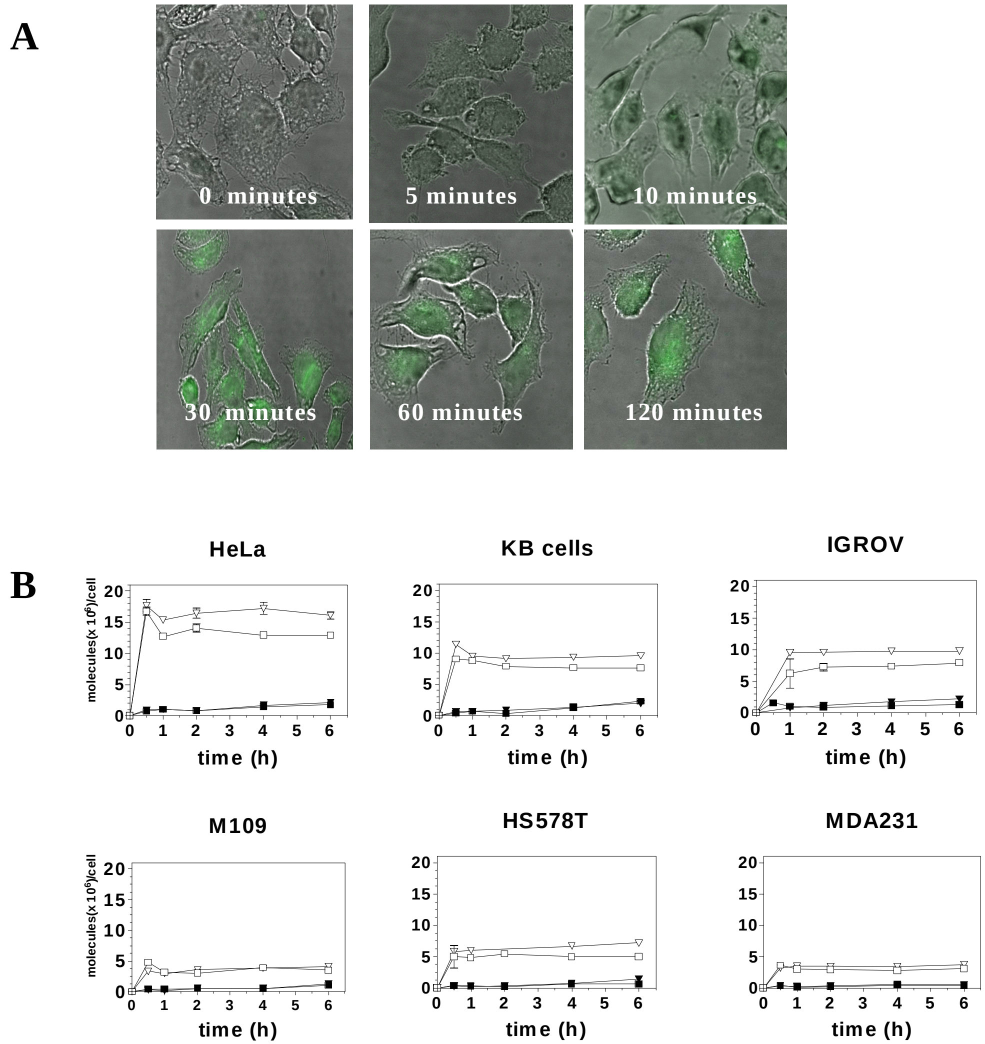

A
0 minutes
5 minutes
10 minutes
30 minutes
60 minutes
120 minutes
B
HeLa
KB cells
IGROV
M109
HS578T
MDA231
molecules(x 10^6)/cell
time (h)

Figure 2

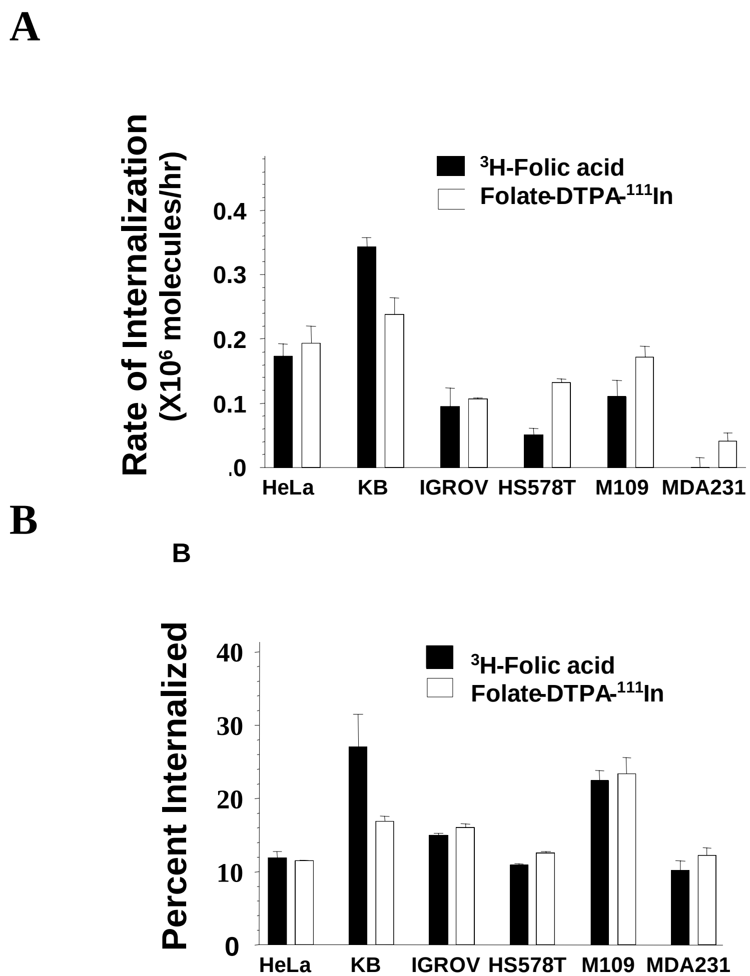

A
Rate of Internalization
(X10^6 molecules/hr)
0.4
0.3
0.2
0.1
.0
^{3}H-Folic acid
Folate-DTPA-^{111}In
HeLa
KB
IGROV
HS578T
M109
MDA231
B
B
Percent Internalized
40
30
20
10
0
^{3}H-Folic acid
Folate-DTPA-^{111}In
HeLa
KB
IGROV
HS578T
M109
MDA231

Figure 3

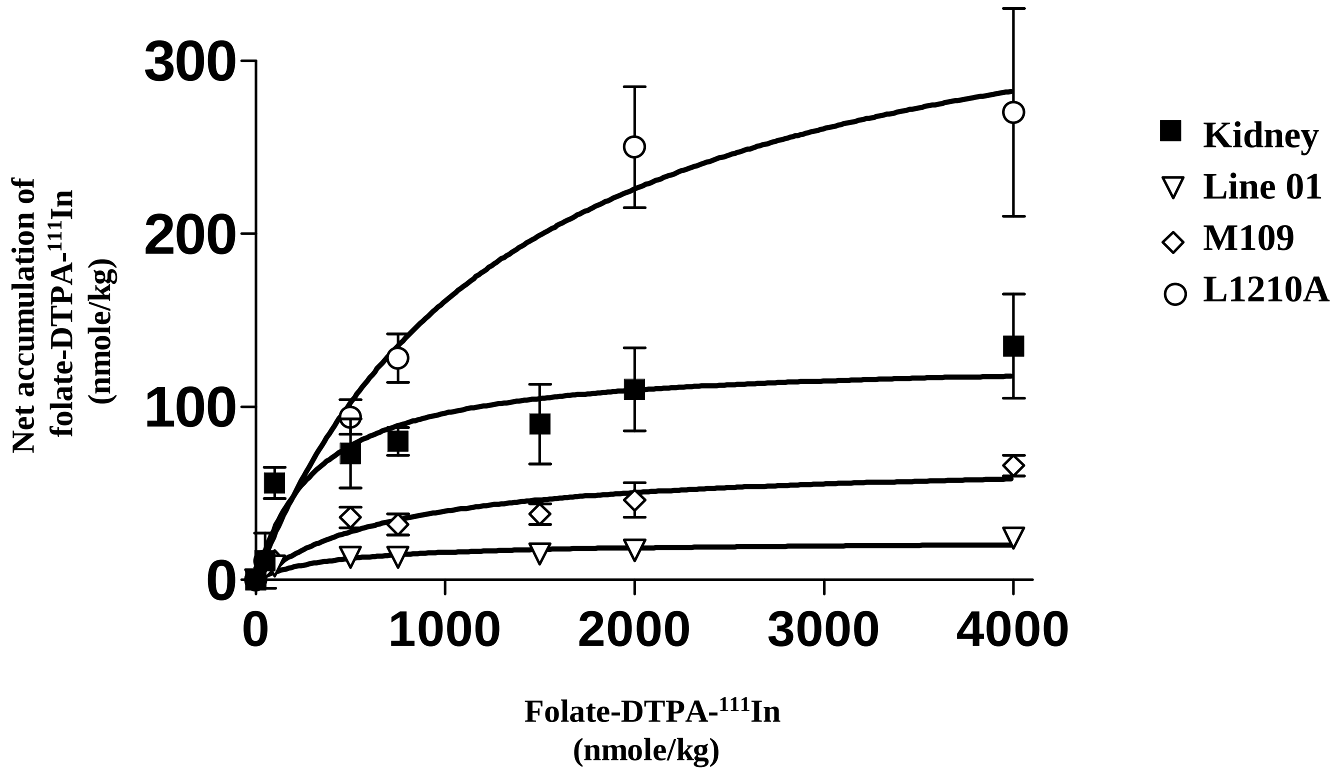
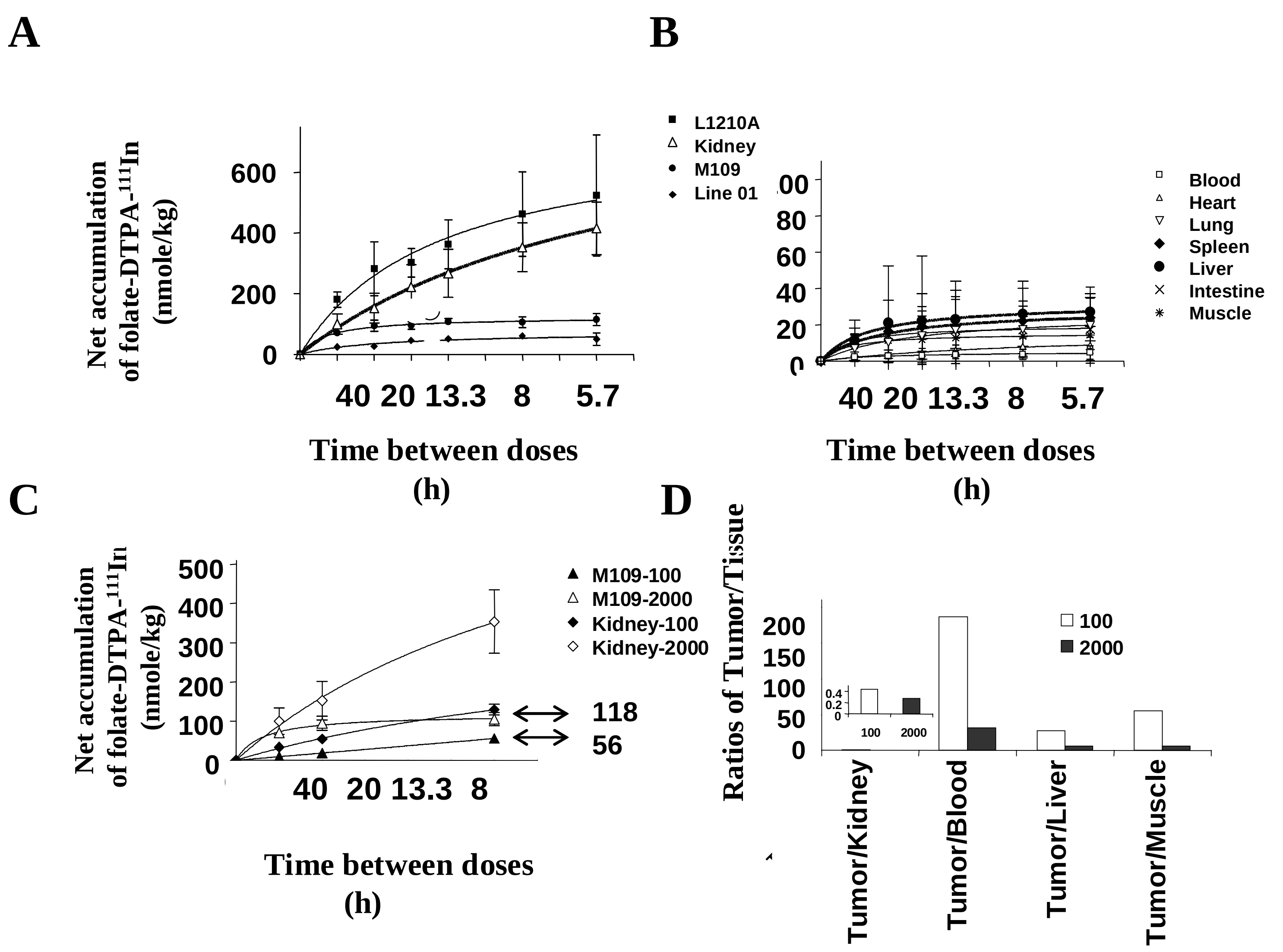

300
200
100
0
0
1000
2000
3000
4000
Net accumulation of folate-DTPA-111In (nmole/kg)
Folate-DTPA-111In (nmole/kg)
Kidney
Line 01
M109
L1210A

Figure 4


A
Net accumulation of folate-DTPA-111In (nmole/kg)
0
200
400
600
40 20 13.3 8 5.7
Time between doses (h)
L1210A
Kidney
M109
Line 01
B
0
20
40
60
80
.00
40 20 13.3 8 5.7
Time between doses (h)
Blood
Heart
Lung
Spleen
Liver
Intestine
Muscle
C
Net accumulation of folate-DTPA-111In (nmole/kg)
0
100
200
300
400
500
40 20 13.3 8
Time between doses (h)
M109-100
M109-2000
Kidney-100
Kidney-2000
118
56
D
Ratios of Tumor/Tissue
0
50
100
150
200
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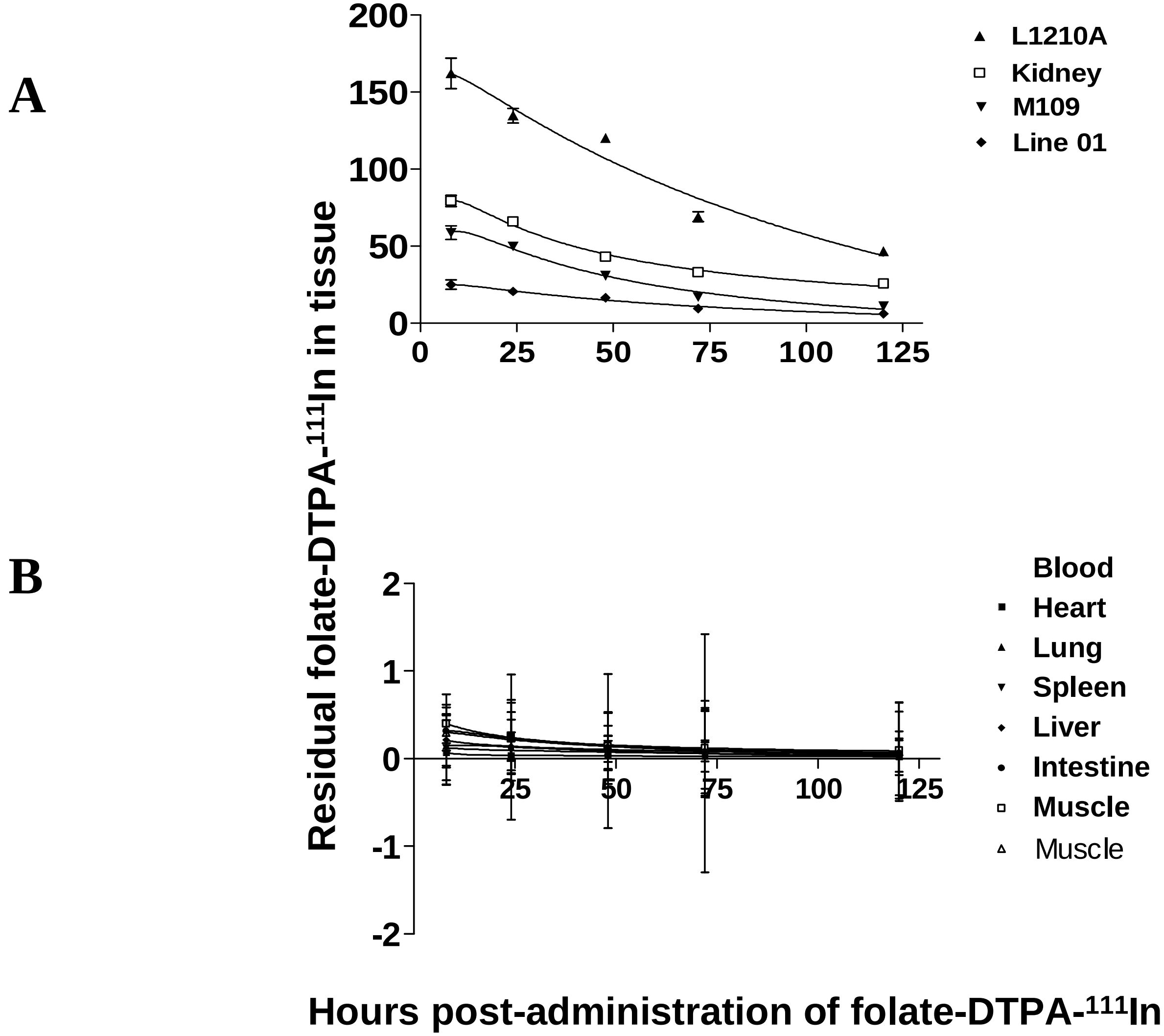
0.2
0
100
2000
Tumor/Kidney
Tumor/Blood
Tumor/Liver
Tumor/Muscle

Figure 5


A
B
200
150
100
50
0
0
25
50
75
100
125
L1210A
Kidney
M109
Line 01
2
1
0
-1
-2
25
50
75
100
125
Blood
Heart
Lung
Spleen
Liver
Intestine
Muscle
Muscle
Residual folate-DTPA-111In in tissue
Hours post-administration of folate-DTPA-111In