TITLE: Impact of High and Low Folate Diets on Tissue Folate Receptor Levels and Anti-tumor Responses Towards Folate-drug Conjugates

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Non-standard abbreviations: FR, folate receptor; RBC, red blood cell; FA, folic acid; FFRPMI, Folate-free RPMI media; sFBP, bovine soluble milk folate binding protein; PBS-T, PBS containing 0.05% Tween-20.
ABSTRACT

Herein we present a detailed analysis on the effects of feeding laboratory mice both high and low folic acid (folate)-containing diets as related to associated changes in serum and red blood cell (RBC) folate levels, tissue-derived folate receptor levels, and the ability of folate-drug conjugates to bind and effectuate activity against folate receptor (FR)-positive tumor xenografts. Our data show that serum and RBC folate concentrations sharply drop immediately after mice are switched to low folate diets; however, both parameters reach steady-state, “human-like” levels after 6 weeks. Interestingly, tissue-related folate binding capacities were also lowered during the dietary modulation period, while the net uptake of a radiolabeled folate conjugate was simultaneously increased 2.6- and 5-fold in FR-positive kidney and tumor tissue, respectively. Finally, the performances of several clinically- and preclinically-relevant folate-drug conjugates were evaluated against tumors in mice that were fed high or low folate diets. Except when administered at a dose level 6-fold less than that required to saturate endogenous FRs, no significant loss of anti-tumor activity was observed. From these findings we conclude that lowering the dietary intake of folates in mice has little impact on the biological activity of repetitively-dosed folate-targeted agents; but, low folate diet regimens will reduce serum and RBC folate levels down to levels that more closely approximate the normal human ranges.
INTRODUCTION

Folic acid (FA, folate, vitamin B9) is an essential nutrient required by all living cells for proper metabolic maintenance of 1-carbon pathways as well as for nucleotide biosynthesis (Clifford et al., 1998). Hence, acquisition of folates is extremely important for maintaining the viability of both normal and proliferating (e.g. cancer) cells. Many have studied the impact of modulating dietary FA intake in rodents on the activities of various chemotherapeutic agents, including 5-fluorouracil, mitomycin C, cytosine arabinoside, doxorubicin, cyclophosphamide, and the antifolates aminopterin, methotrexate, dideazatetrahydrofolic acid and pemetrexed (Mendelsohn et al., 1996; Raghunathan et al., 1997; Worzalla et al., 1998; Branda et al., 2002). In some cases, lowering the intake of folate was found to potentiate anti-tumor activity, whereas in others (e.g. antifolates), greater toxicities were observed compared to when animals were fed high folate diets. Such preclinical modeling had proved to be, in part, useful for guiding the clinical development of some agents (Azzoli et al., 2007); however, increasing dietary folate levels has also been met with problems, since rapid hematologic and clinical relapses were noted in some leukemia patients (Lennard et al., 1986).

At the cellular level, uptake of folates mainly occurs by two independent transport mechanisms, namely (i) transport of reduced folates by a carrier-mediated, low-affinity but high capacity anion-transport protein (Antony, 1992), or (ii) by an active endocytic process mediated by the high-affinity membrane-bound glycoprotein receptor, commonly referred to as the folate binding protein or the folate receptor (FR) (Kamen et al., 1988; Antony, 1996). The FR is a known biomarker protein that’s expressed in high quantities by many primary and metastatic cancers (12-14), but not on most normal cells (15). Recognizing this attribute, we have focused
our efforts on the development of small molecule targeting systems, based on FA, to deliver covalently-linked therapeutic and diagnostic imaging agents to cells that express the FR (Leamon and Low, 1991; Leamon et al., 2002; Leamon and Reddy, 2004; Reddy et al., 2004). In recent years, our lab and research collaborators have reported on the preclinical pharmacology results for a variety of FA-drug conjugates, including those constructed with mitomycin C, desacetylvinblastine monohydrazone, epothilone B and the maytansinoid DM1 (Leamon et al., 2005; Leamon et al., 2006; Reddy et al., 2006; Leamon et al., 2007a; Leamon et al., 2007b; Reddy et al., 2007a; Reddy et al., 2007b; Covello et al.). In each case, strong evidence was presented for FR-mediated cytotoxicity and \textit{in vivo} anti-tumor effect. Importantly, three related agents (EC145, EC0225 and BMS-753493) are currently being evaluated for safety and activity in multiple Phase 1 and 2 clinical trials.

Supportive preclinical data for each of the aforementioned compounds were collected using animals that had been acclimated on a low folate diet for two or more weeks prior to test article administration. This technique is commonly done because normal rodent laboratory chow contains high levels of FA (~6 mg/kg chow) which allow for excessively high serum folate concentrations to persist (Mathias et al., 1996). Importantly, such supra-physiological levels of FA in the sera of mice were predicted to decrease the uptake of FA-based radiodiagnostic agents in FR-expressing tumor tissue; therefore protocols using short dietary modulation with non FA-supplemented chows were developed for the purpose of lowering mouse serum folate levels down towards the normal human range (Mathias et al., 1996). As a consequence of that finding, nearly all \textit{in vivo} studies reported for FA-drug conjugates have involved the use of these low FA diets. What is not known, however, is whether the anti-tumor activities of frequently-
administered FA-drug conjugates would truly be compromised in mice that were maintained on
the more common high folate-containing diets.

In our current study, we present a detailed analysis of modulating dietary folate levels in
laboratory mice on the effects of serum and red blood cell (RBC) folate levels, tissue-derived
folate receptor levels, and the ability of both clinically-and preclinically-relevant FA-drug
conjugates to bind and effectuate activity against FR-positive tumor xenografts.

METHODS

Materials.

Folate-free RPMI media (FFRPMI) and PBS were obtained from Gibco, Grand Island,
NY. Bovine soluble milk folate binding protein (sFBP) was purchased from Scripps (item
#F0524). 3H-thymidine (71.9 Ci/mmol) and 3H-folic acid (30 Ci/mmol) were purchased from
Moravek Biochemicals, Brea, CA. Sera samples were obtained from Harlan (Indianapolis, IN;
Balb/c mice, Lewis rats and New Zealand White rabbits), Bioanalytical Systems Incorporated
(Evansville, IN; Beagle dog and Cynomolgus monkey) and human donors. All test diets were
produced by Harlan Teklad, Madison WI and included (i) Teklad Diet #2018S Global 16%
Protein Rodent Diet (Sterilizable), (ii) Test Diet #00434 which is the standard chow devoid of
added folic acid, and (iii) TD#00633, the folate-free chow supplemented with 0.5 mg/kg of
leucovorin. All other common reagents were purchased from Sigma (St. Louis, MO) or other
major suppliers.
Test Articles.

EC20 (pteroyl-γ-D-Glu-βDpr-Asp-Cys), EC145 (folate-desacetylvinblastinehydrazide) and EC0305 (folate-tubulysin B hydrazide) were produced by Endocyte, Inc. (West Lafayette, IN). Their syntheses, purifications and analytical characterizations have been described in detail elsewhere (Leamon et al., 2002; Leamon et al., 2005; Leamon et al., 2006; Vlahov et al., 2006; Vlahov et al., 2007; Vlahov et al., 2008).

Mouse Animal Housing/Conditions.

Four to 5 week old female BALB/c or nu/nu mice (Balb/c background) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed for study in polycarbonate shoebox cages with shredded aspen beddings at Endocyte’s Animal Facility for duration of study. Bedding was replaced twice a week in addition to fresh cages biweekly by a qualified technician. The diet and drinking water (municipal) were provided ad libitum throughout the study periods. Mice were housed in an environmentally controlled room with temperature settings ranged from 70°F to 74°F and relative humidity ranged from 30% to 70%. Light cycles were set to provide a 12 h light/12 h dark photoperiod. Test diets were provided to mice for duration of study. Mice were euthanized at end of diet duration using carbon dioxide. All housing, care, and procedures were followed according to Endocyte-approved animal care and use protocols.
Serum and Red Blood Cell (RBC) Total Folate Quantitation.

Mouse blood was obtained by cardiac puncture as part of a terminal procedure. For whole blood, samples were collected in pediatric sized K$_2$EDTA-coated tubes (BD Microtainer #365974). For sera, blood was collected in uncoated tubes and allowed to sit at room temperature for half an hour. Samples were spun two consecutive times at 6600 x g at 4°C for 10 min. All samples collected on a particular day were then shipped for analysis (overnight on cold packs) to Ani-lytics, Inc (Gaithersburg, MD).

Methods used for measuring serum and RBC folates were proprietary to Ani-lytics, Inc. Briefly, total folate levels in sera or RBC samples were measured using Dualcount reagent kit (DPC #KDSP) supplied by Diagnostic Products, Los Angeles, CA. Whole blood samples were lysed in 1% ascorbic acid solution. Whole blood hemolysate samples, serum samples (diluted in ascorbate solution) and standard folate samples were then treated with DTT plus an $^{125}$I–folic acid tracer followed by an extraction step performed at alkaline pH. Folates were extracted by mixing milk folate binding protein immobilized to a solid support. Following a brief centrifugation and removal of the supernatant, bound radioactivity was measured with a gamma counter. The total folates in the sample were quantitated by comparison with a standard curve.

Folate Receptor Assay.

Tissue FR levels were quantitated according to a previously reported procedure (Parker et al., 2005). Briefly, membrane pellets were isolated from homogenized tissue samples and then solubilized in an octyl-β-D-glucopyranoside containing buffer. Aliquots of solubilized membranes were placed inside each of two Microcon-30 microconcentrators (30,000 molecular

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weight cut-off; Millipore Corporation) and then acid-stripped to remove endogenously bound folates. $^3$H-folic acid binding reagent (in the presence or absence of a 1000-fold excess of unlabeled folic acid) were added to the appropriate concentrators. Following a 10 min incubation at room temperature, the concentrators were washed, and the retentates containing the solubilized FRs were recovered from the membrane surface of the microconcentrators. The samples were then counted in a liquid scintillation counter (Packard Bioscience Co.). CPM values were converted to picomole of FR based upon the CPM of a known standard, and the final results were normalized with respect to the sample protein content.

**Relative Binding Assay Using Immobilized Folate Binding Protein.**

One hundred microliters of a sFBP solution (10 μg/mL in PBS) were added to each well of a Reacti-Bind® microtiter plate. Plates were incubated at 4°C overnight and then washed 3 times with cold PBS containing 0.05% Tween-20 (PBS-T). Plates were equilibrated to room temperature, blocked for 1 h at room temperature with 100 μL/well of freshly-prepared PBS-T containing 0.2% gelatin, and then washed 3 additional times with PBS-T. Undiluted serum samples or BSA (43 mg/mL in PBS) were added to FBP-coated ELISA plates for 2 h at 37°C. Wells were washed 3 times with PBS, and then 100 μL of 100 nM $^3$H-FA in PBS (per well) were added in triplicate. Plates were incubated at 37°C for 30 min with gentle shaking and then rinsed 3 times with PBS-T. Bound $^3$H-FA was then stripped from the plates with 100 μL of acid-saline solution (20 mM sodium acetate, pH 3.0) for up to 20 min at room temperature, and the acidic samples were transferred into individual scintillation vials containing 3 mL of scintillation
cocktail. Values are expressed as the percent maximum binding relative to PBS-treated (control) wells, which bound 107,671 ± 3314 DPM.

**FolateScan (99mTc-EC20) Uptake in KB Tumors and Kidneys of nu/nu Mice.**

An EC20 kit was used for the preparation of radioactive drug substance (Leamon et al., 2002). Each kit contains a sterile, non-pyrogenic lyophilized mixture of 0.1 mg of EC20, 80 mg of sodium α-D-glucoheptonate dihydrate, 80 µg of tin (II) chloride dihydrate, and sufficient sodium hydroxide or hydrochloric acid to adjust the pH to 6.8 ± 0.2. Chelation of 99mTc to EC20 was done by injecting 1 mL of Sodium Pertechnetate 99mTc Injection (<50 mCi) into this vial and heating it for ~ 18 min in a boiling water bath. Samples of the 99mTc-EC20 solution were analyzed for radiochemical purity using an HPLC system consisting of a Waters 600E Multisolvem Delivery System and 490 UV detector, a Bioscan FC-3200 radiodetector, Laura v1.5 radiochromatogram software, and a Waters Nova-Pak C18 (3.9 x 150 mm) column. Injected samples were eluted isocratically using an aqueous mobile phase containing 20% methanol and 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The radiochemical purity of 99mTc-EC20 was greater than 90% (Leamon et al., 2002).

Female nu/nu mice (Balb/c background; 6-7 weeks of age; Harlan, Indianapolis, IN) were maintained on the folate-rich or folate-free diets for a total of three weeks prior to the biodistribution experiment. FR-positive KB tumor cells (1 x 10⁶ cells per animal) were inoculated in the subcutis of the dorsal medial area of the right axilla. Stock 99mTc-EC20 solutions containing 100 µg of agent per milliliter were prepared on the day of use. Mice with 300-500 mm³ KB tumors received a 99mTc-EC20 dose of 67 nmol/kg in 200 µL of sterile PBS via a tail vein. Four hours post injection, animals were sacrificed by CO₂ asphyxiation and
dissected. Tumors and kidneys were removed, weighed and their radioactivity content measured in an automatic gamma counter to determine $^{99m}$Tc distribution. Uptake of the radiopharmaceutical, expressed as percent injected dose of wet weight tissue (%ID/g), was calculated by reference to standards prepared from dilutions of the injected preparation (Reddy et al., 2004).

**Tumor Model and Therapy.**

Six to eight week-old female $nu/nu$ mice (Charles River, Wilmington, MA) were maintained on a standard 12 h light-dark cycle for the duration of the experiment. Mice used in these studies were fed defined chows beginning 2 weeks before tumor implantation and maintained throughout the experiment. FR-positive KB cells ($1 \times 10^6$ per $nu/nu$ mouse) in 100 µL of folate-free RPMI 1640 were injected in the subcutis of the dorsal medial area. Tumors were measured in two perpendicular directions every 2-3 days using a caliper and their volumes calculated as $0.5 \times L \times W^2$, where $L =$ measurement of longest axis in mm and $W =$ measurement of axis perpendicular to $L$ in mm. Dosing solutions were prepared fresh each day in PBS and administered through the lateral tail vein of the mice. Importantly, dosing was initiated when the s.c. tumors were between $133 \pm 26$ mm$^3$ in volume.

**RESULTS**

**Common Laboratory Animals Have High Serum Folate Levels.**

Humans are known to have low serum folate levels, typically in the 20 nM range (Venn et al., 2002). For comparison, sera obtained from 5 commonly used laboratory species, along with freshly isolated human donor sera, were tested for total folate content using a commercial
radioimmunoassay. As shown in Fig. 1, sera taken from Balb/c mice were found to contain the highest level of folates at nearly 250 nM. Importantly, this level is approximately 10-fold higher than that measured in human sera (see below). Serum folates from Lewis rats and New Zealand White rabbits were also very high (range 130-180 nM), whereas the folate levels in Beagle dog and Cynomolgus monkey sera were in the same range as that measured in normal human sera (~25 nM). These data collectively show that the choice of animal model could be critical when studying the biological effects of folate-based compounds.

**Low Folate Diets Decrease Serum and Red Blood Cell Total Folate Levels.**

Since (i) mice are most commonly used for evaluating the performance of most anticancer agents, and (ii) the high serum folate levels in mouse sera (Fig. 1) could negatively impact the performance of folate-drug conjugates used for cancer therapy, we chose to study the impact of placing mice on low folate chows on the levels of folates in the sera as well as red blood cells (RBCs). Importantly, the former parameter is a measure for short-term folate status, whereas the latter parameter is a measure for long-term folate status. As shown in Fig. 2A, pre-study serum folate levels for mice ~ 8 weeks of age were again very high (241 ± 7 nM). Serum folate levels did actually drop somewhat (to 180 ± 63 nM) while maintaining these mice on high folate chow for 18 weeks, likely due to natural age-related changes. In contrast, serum folate levels abruptly fell in mice that were fed a low folate chow, and the time-related decrease was independent of whether or not the chow was supplemented with 0.5 mg/kg of leucovorin, which is a reduced form of folic acid that does not significantly interfere with FA-drug targeting (Reddy et al., 2004). Furthermore, 6 weeks after the start of these “low” folate diets, serum folate levels appeared to stabilize, because the concentration remained steady at ~ 20 nM during
weeks 6 through 18. The cause for this stabilization is merely speculative, but it could be due to the low amount of folates supplied naturally by the intestinal flora of the mice (Klipstein and Lipton, 1970). Regardless, the data in Fig. 2A indicate that dietary modulation can lower the serum folate levels of laboratory mice down to nearly the same levels as humans within a short period of time.

In addition to serum folate, RBC folate levels were also found to abruptly drop when mice were fed low folate chows. As shown in Fig. 2B, pre-study RBC folate levels for mice ~ 8 weeks of age were initially very high (4329 ± 44 nM), but they fell to ~ 1000 nM after 6 weeks on the low folate chows. Interestingly, these RBC folate levels are within the 347 to 1167 nM range measured in humans (Pfeiffer et al., 2005). Thus, the data in Fig 2 show that both serum and RBC folate levels decrease in mice to concentrations which closely resemble that found in humans when the animals are fed low folate diets for at least 6 weeks.

**Tissue Folate Receptor Levels Decrease When Animals Are Fed Low Folate Chows.**

Besides serum and RBC changes, we also examined the effects of dietary folate modulation on major organ tissue-derived FRs. As shown in Fig. 3A, negligible FR levels [i.e. > 2.5 pmol/mg membrane protein; (Parker et al., 2005)] were found in all tissues pre-study except for kidney. Renal cortex is known to be rich with functional FR expression (Holm et al., 1992), therefore this finding was expected. However, following 18 weeks of eating low folate chow, FR levels surprisingly did not rise in any murine tissue examined; instead, FR levels actually decreased to undetectable levels in all tissues except, again, for the kidney.

Further examination of the dietary effects on kidney FR expression is shown in Fig. 3B. Kidney FR levels were measured at 12.31 ± 1.45 pmol/mg when the mice were ~ 8 weeks of age,
but these levels naturally dropped to 3.28 ± 0.19 pmol/mg while maintaining the mice on high folate chow for 24 consecutive weeks (again, probably due to natural age-related changes). Interestingly, feeding the younger mice low folate chows was found to cause a rapid drop in kidney-associated FR levels by as much as 88% to ~1.5 pmol/mg within 6 weeks. Further decline in FR levels was not observed up to the 24 week period. This relationship between low folate intake and kidney FR expression seems paradoxical, especially since kidney FRs are believed to function as “salvage” receptors for this easily-filtered vitamin (Birn et al., 1997). But, this effect was observed by others before (see discussion) (Gates et al., 1996; Da Costa et al., 2000).

**High Serum Folate Levels in Mice Interferes with $^3$H-FA Binding to the FR In Vitro.**

To ascertain the biological significance of lowering serum folate levels through dietary modulation, we tested the ability of radiolabeled folate to bind to its receptor in the presence of different test sera. Thus, normal Balb/c and nu/nu (Balb/c background) mice were fed either high folate or low folate chows for 3 weeks. Sera obtained from these animals were then tested along with pooled human sera and an albumin/PBS control solution for the ability to block $^3$H-FA from binding to immobilized folate binding protein. As shown in Fig. 4, maximal $^3$H-FA binding was measured in wells treated with either the albumin control or 100% pooled human sera. Serum samples obtained from the “high folate” animals were found to block $^3$H-FA binding by >80%. In contrast, serum samples obtained from the “low folate” animals had blocked $^3$H-FA binding by no greater than 23%. Taken together with the data in Figs. 1 and 2, these results confirm that (i) mice fed standard laboratory chows have high serum folate levels that can competitively block the binding of folates to the FR, and (ii) short dietary modulation with low folate chows
will reduce serum folates to “human-like” (Fig. 2) which minimally interfere with ligand binding to the FR.

**Folate-targeted Radiopharmaceutical Tissue Retention Increases When Animals Are Fed Low Folate Chows.**

We next explored the effect of modulating dietary folate levels on the ability of $^{99m}$Tc-EC20, a folate-based radiopharmaceutical (Leamon et al., 2002; Reddy et al., 2004; Fisher et al., 2008), to target FR+ tissues *in vivo*. In addition to kidney, which is the predominant FR+ tissue in mice (Fig. 3), we also implanted and assayed FR+ KB tumors in the test animals for $^{99m}$Tc-EC20 retention to better assess the dietary effects on FR targeting. As shown in Table 1, $^{99m}$Tc-EC20 retention was prominent in the kidneys, but low in the tumors of mice fed the high folate diet. However, we measured a 2.6- and 4.9-fold increase in kidney and tumor retention, respectively, when the mice were fed the low folate diet. Notably, the former result was nearly identical to that observed for folic acid’s kidney retention in a similar study (Gabizon et al., 2003). Collectively, these results indicate that while access to FR+ tissues is not completely blocked for animals on high folate diets, upwards of 5 times more folate-labeled compounds can be delivered to these FR+ tissues if the laboratory chow is not heavily supplemented with folic acid.

**A High Folate Diet Has Minimal Impact on the Anti-tumor Response Towards Potent Folate-Drug Conjugates.**

There are currently three distinct folate-chemotherapeutic drug conjugates being clinically evaluated in multiple phase 1 and 2 oncology trials across the U.S., and clinical
development is being planned for at least two additional agents in the near future [(Leamon et al., 2007a; Reddy et al., 2007a; Sausville et al., 2007; Covello et al., 2008) and Endocyte Inc.]. Related to this study, EC145 and EC0225 are examples of clinically evaluated conjugates, whereas EC0305 represents one that is under consideration for development. The impact of feeding mice high folate diets on the performances of these potent therapeutic agents was next evaluated using the KB nu/nu model. In our first experiment, tumor-bearing mice feeding on the low folate chow had endured complete responses and remained tumor-free for > 90 days after being briefly treated with 2 µmol/kg of EC145 or 1 µmol/kg of EC0225 (Fig. 5). Interestingly, EC0225’s performance was not altered if the mice had been fed the high folate chow. However, tumors in one of five mice in the EC145 cohort did recur while on the high folate diet.

In a second experiment, we evaluated the impact of each diet on the performance of increasing dose levels of EC0305. This agent is a potent folate conjugate of the microtubule inhibitor, tubulysin B monohydradize (Reddy et al., 2008; Vlahov et al., 2008). As shown in Fig. 6A, 100% of the animals fed the low folate chow and treated with 1 or 2 µmol/kg EC0305 had endured complete responses and remained tumor-free for > 90 days. Mice in the low 0.5 µmol/kg cohort also responded well, with 4/5 having complete responses (2 of which remained tumor-free at the end of study) and 1/5 having a partial response. Similar to what was observed for EC145 and EC0225 (Fig. 5), maintaining mice on the high folate diet had no negative consequences for animals treated at ≥ 1 µmol/kg EC0305, since each of these animals remained tumor-free for > 90 days (Fig. 6B). But, mice that were fed the high folate diet and treated with the low 0.5 µmol/kg dose level did not fare as well as their counterparts which had been maintained on the low folate chow. Although a small anti-tumor signal was present during this brief 2 week treatment (on the high folate diet), we observed only 1 partial responder in that cohort (Fig. 6A).
Collectively, the data presented in Figs. 5 and 6 suggest that a high folate diet has a small but measurable impact on the anti-tumor response towards potent folate-drug conjugates, and such negative effects on pharmacological responses may be overcome by slightly increasing the dose of the administered folate-drug agent.

**DISCUSSION**

As with any promising therapeutic approach, use of relevant preclinical models becomes paramount to the successful identification of clinical candidates and, ultimately, the development of effective products for human use. Our laboratory has been actively researching and now developing FA-targeted drug therapies for nearly 20 years. We recognized long ago that high serum folate concentrations, like that found in the sera of laboratory rodents, could potentially block the binding of FA-drug conjugates to FR-positive tissues, e.g. tumors (Mathias et al., 1996). Because of that finding, the vast majority of “folate-targeted” pharmacological studies published to date had involved the use of animals that were acclimated to some form of low folate chow. Since we have been fortunate to witness the start of clinical testing for a number of folate-based agents, the impact that a patient’s diet might have (i.e. net folate intake) on the performance of these novel agents has been called into question. Therefore, the purpose of our current work was to directly examine such dietary effects using well-known animal models and to determine if changes in dietary folate could alter the anti-tumor effect of some clinically- and preclinically-relevant compounds.

Dietary modulation, using a standard rodent laboratory chow devoid of supplemental FA, was found to quickly lower both serum and RBC folates in mice to concentrations that better approximate those levels found in human sera. In fact, “human-like” folate levels were reached
in the mice within 6 weeks (Fig. 2). At that point, and at least up to 18 weeks thereafter, serum and RBC folate levels reached steady state levels. This observation was important, because our tumor therapy protocols allow for responding animals to remain on study for up to 120 days, suggesting that animal nutrition and welfare should never be compromised. Notably, therapy with folate-drug conjugates typically begins 2 or 3 weeks after the start of the low folate diet ([Leamon et al., 2005; Leamon et al., 2006; Reddy et al., 2006; Leamon et al., 2007a; Leamon et al., 2007b; Reddy et al., 2007a; Reddy et al., 2007b] and Figs. 5 and 6). Since serum and RBC folate concentrations do not reach their neo-homeostatic levels until week 6, one may argue that better therapeutic outcomes could result if therapy were delayed until that point. However, since (i) housing costs are expensive, (ii) serum folate levels are 75% lower in mice after only 2 weeks on the low folate diet, and (iii) tumor-free responses are often observed with folate-drug therapies when initiated at that earlier time, we believe it is not necessary to delay the start of in vivo studies until that 6 week point.

Throughout our investigation, we noted that while mice were maintained on the low folate diet, $^{99m}$Tc-EC20 uptake in the kidney was observed to increase 2.6-fold; yet, their kidney FR levels concomitantly dropped by 88% (compare Table 1 and Fig. 3, respectively). The increase in $^{99m}$Tc-EC20 kidney uptake can be explained by the presence of empty FRs in that tissue relative to a minuscule amount under high dietary folate conditions. The massive loss of kidney FRs observed in response to low folate diets, while intriguing, has actually been observed by others before (Gates et al., 1996; Da Costa et al., 2000), but the cause for this dramatic change was not defined. Although this apparent paradox is not the subject of this report, we propose that the diet-induced drop in kidney FR levels may be the result of FR naturally “shedding” into the blood, in ligand-bound form, to promote uptake and retention of the ligand:FR complex by the
liver. In other words, such a process could serve as a means for better salvaging folates under more limiting conditions. Certainly, it is known that liver uptake of FA is dramatically increased when injected into circulation as a pre-complex with the soluble form of the FR (Fernandes-Costa and Metz, 1979; Rubinoff et al., 1981). Plus, we have observed a near 500% increase of $^{99m}$Tc-EC20 uptake in liver, also when injected into circulation as a FR pre-complex (Endocyte Inc., unpublished results). Because (i) the liver is the recognized storage organ for folates (Corrocher et al., 1974), and (ii) we can not detect soluble FR (by ELISA) in the urine of mice on low folate diets (Endocyte Inc., unpublished data), we interpret these results to suggest that the FR could be a conduit by which renally-trapped folates leave the kidney for delivery and storage in the liver. Admittedly, more studies are needed to confirm this hypothesis.

The most interesting observation reported here, though, is that a high folate diet appears to have little effect on the performance of potent folate-drug conjugates in mice. Except at a dose level that is ~ 6-fold lower than that which promotes FR saturation (Reddy et al., 2007a), maximal anti-tumor effect was observed in mice fed the high folate diet and then treated with clinically (EC145 and EC0225) and preclinically (EC0305) relevant drug conjugates (see Figs. 5 and 6). Admittedly, the data in Table 1 do argue that the net tumor uptake from a single administered dose is significantly compromised under high folate conditions. However, we believe that the strong therapeutic results, as presented in Figs. 5 and 6, are not surprising given that the active drug moieties in the tested conjugates are inherently very potent (e.g. Vinca, mitomycin, tubulysin), and a complete course of therapy involved 6 total doses spread over 2 weeks. Admittedly, it is certainly possible that more pronounced negative therapeutic effects could result when conjugates of lower potency are tested in mice being fed high folate diets.
In summary, we have found that feeding mice high folate diets leads to elevated, supra-physiological serum and RBC folate concentrations, the former having significant effects on lowering the amount of available folate binding sites on FR-positive cells and tissues. The consequences of this finding, in regards to tumor targeting, are that higher dose levels of a folate-drug conjugate may be needed to afford maximal anti-tumor responses in the presence of high serum folate concentrations. Since the sera of the “high-folate” mice contain nearly 10 times the amount of folate to that found in human sera, these findings may only be relevant to preclinical models. However, future studies involving the effects of human serum folate levels (and perhaps vitamin supplementation) on the performance of folate-drug conjugate therapies are warranted to better determine the full translational impact.

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FOOTNOTES

1 No significant changes were noted in $^{99m}$Tc-EC20 retention by all other major (FR-negative) organs.
LEGENDS FOR FIGURES

Fig. 1. **Serum Folate Levels of Various Species (no diet modulation).** Animal sera were obtained from donors (3 per specie) maintained on standard laboratory diets.

Fig. 2. **Time-dependent Effects of Low Folate Diets on Balb/C Mouse Serum and Red Blood Cell (RBC) Total Folates.** Panel A, serum folates. Panel B, RBC folates. (■) High folate diet; (●) Low folate diet; (▼) Low folate diet supplemented with 0.5 mg/kg leucovorin.

Fig. 3. **Time-dependent Effects of Low Folate Diets on Tissue Folate Receptor Levels.** Panel A, FR levels in major organ tissues during the first (open bars) and 18th (black bars) week following the onset of the low-folate diet. Panel B, FR expression in kidney with respect to time on low folate diet. (○) High folate diet; (●) Low folate diet; (▼) Low folate diet supplemented with 0.5 mg/kg leucovorin.

Fig. 4. **Effect of Serum-derived Folates on \(^3\)H-FA Binding to FBP Plates.** Samples were added to FBP-coated ELISA plates for 2 h at 37°C. Following several washes, ice-cold 100 nM \(^3\)H-FA was added to each well for 30 min to bind to any unoccupied FBP sites. Bound \(^3\)H-FA was then stripped from the plates using acid-saline, and samples were counted for total radioactivity. Values are expressed as the percent maximum binding relative to PBS-treated (control) wells. All sera samples were tested as undiluted
solutions. The albumin control was 43 mg/mL in PBS. HFD, high folate diet; LFD, low folate diet.

Fig. 5. **Effect of High Folate and Low Folate Diets on the Anti-tumor Response to EC145 and EC0225 Therapies.** KB tumor cells (1 x 10^6) were implanted s.c. into nu/nu mice, and eleven days later, mice were randomized and treatments given following a three times per week, 2 week schedule. (■) untreated mice; (○,●) mice treated with 2µmol/kg EC145 on high folate and low folate diets, respectively. (△,▲) mice treated with 1 µmol/kg EC0225 on high folate and low folate diets, respectively. The dotted vertical line represents the day of final dosing (Day-22). Each cohort contained 5 animals, and data shown represent mean ± SEM.

Fig. 6. **Effect of High Folate and Low Folate Diets on the Dose-responsive Anti-tumor Responses to EC0305 Therapy.** KB tumor cells (1 x 10^6) were implanted s.c. into nu/nu mice, and eleven days later, mice were randomized and treatments given following a three times per week, 2 week schedule. (■) untreated mice. Panel A, (●,▲,▼) mice on low folate diets treated with 0.5, 1 and 2 µmol/kg EC0305, respectively. Panel B, (○,△,▽) mice on high folate diets treated with 0.5, 1 and 2 µmol/kg EC0305, respectively. The dotted vertical line represents the day of final dosing (Day-22). Each cohort contained 5 animals, and data shown represent mean ± SEM.
Table 1. Retention of $^{99m}$Tc-EC20 in FR-Positive Kidney and Tumor Tissue. KB tumor-bearing mice were placed on the specified diets for 3 weeks prior to receiving $^{99m}$Tc-EC20. FR, folate receptor. %ID, percent injected dose per gram weight-weight tissue.

<table>
<thead>
<tr>
<th>FR+ Tissue</th>
<th>Diet</th>
<th>$^{99m}$Tc-EC20 Tissue Retention (% ID/g)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Folate</td>
<td>25.8 ± 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No Folate</td>
<td>66.7 ± 15.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Tumor</td>
<td>Folate</td>
<td>2.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No Folate</td>
<td>14.3 ± 2.6</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Figure 1

Serum Folate (nM)

Balb/C Mouse  | Lewis Rat  | NZW Rabbit | Beagle Dog | Cynomolgus Monkey | Human

250  | 200  | 150  | 100  | 50  | 0
Figure 2A

Serum Folate (nM) vs. Weeks on Diet

Normal Human Range: 6-37 nM
Figure 4

![Bar chart showing percentage maximum binding for different conditions.](chart.png)
Figure 6B

Tumor Volume (mm$^3$) vs. PTI (days)

B

0/5 tumor-free