Pharmaceutical Research, Vol. 26, No. 6, June 2009 (© 2009)
DOI: 10.1007/s11095-009-9840-3

Research Paper

Properties Influencing the Relative Binding Affinity of Pteroate Derivatives and Drug Conjugates Thereof to the Folate Receptor

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Received December 10, 2008; accepted January 20, 2009; published online February 3, 2009

Purpose. Using *in vitro* competition assays, determine salient chemical features of pteroates and pteroate-drug conjugates which afford high affinity to the folate receptor.
Materials and Methods. Both folate binding protein-coated polystyrene plates and adherent human cell-based assays were used to evaluate the effects of assay temperature and buffer composition on pteroate/pteroate-drug conjugate binding affinity. Following assay selection and optimization, the relative binding affinities of ten vitamers and derivatives as well as seven pteroate-drug conjugates were evaluated.
Results. Compared to polystyrene plates containing immobilized folate binding protein, adherent KB cells were determined to be an equally effective, more desirable source of folate receptor for such analyses. Using the latter method, we discovered that a charged group positioned in close proximity to the pteroate's aryl moiety is critical for retaining high binding affinity. We also found that a diverse set of bioactive small molecule agents can be attached to folic acid in a manner that does not appreciably disturb this vitamin's intrinsic high affinity for the folate receptor. However, conjugation of lipophilic, high protein-binding agents to folate was sometimes found to dramatically reduce affinity, which is a finding that best exemplifies the need for having a reliable *in vitro* assay for determining a compound's RA.
Conclusion. Molecules which bind best to the human folate receptor are those that contain hydrophilic regions distal to the ligand's aryl group, and for drug conjugates, an extended hydrophilic spacer placed in-between the pteroate and drug cargo moieties.

KEY WORDS: cancer; conjugate; folate receptor; ligand affinity.

The folate receptor (FR) is a confirmed tumor-associated membrane protein that binds folic acid (FA) with very high affinity ($K_D \sim 10^{-10}$ M) and transports these bound molecules inside cells via an endocytic mechanism (1–3). The exploitation of this process has been referred to as a molecular Trojan horse approach whereby drugs attached to FA are shuttled inside a targeted FR-positive cell in a stealth-like fashion (4).

For years, the focus of our laboratory has been dedicated towards the synthesis of novel FA conjugates for a variety of clinical applications, including radiodiagnostic imaging, chemotherapy, immunotherapy, and inflammation. Our most recent advances to this field have come in the area of targeted chemotherapy, where two distinct and very potent FA-drug conjugates have entered Phase 1 and 2 clinical testing within the last 3 years (5,6). Likewise, additional FA-drug conjugates are expected to enter clinical testing within the next calendar year (Endocyte, Inc.).

Although it is generally accepted that FA can be conjugated to virtually any molecule to mediate delivery inside FR-positive cells (4,7), not all conjugates can be expected to bind to the FR with the same affinity. In fact large drug payloads that get positioned too close to the FA moiety would be predicted, perhaps in a sterically hindered fashion, to alter the vitamin's ability to enter the binding pocket of the FR. Further, the nature of the drug payload may also be important, because intramolecular hydrogen bonding to the many heteroatoms within FA could yield a poorly-binding conjugate that may not properly orient itself into the FR. Thus, the means to quickly evaluate a conjugate's affinity towards the FR was deemed valuable to our program, for such information could be used in a structure–activity setting to eventually optimize the design of a targeted, bio-active agent prior to the onset of pivotal animal studies.

In this report we disclose the details of an optimized cell-based assay that our laboratory routinely uses to evaluate the affinity of FA-drug conjugates for the FR. We also provide binding data from a series of FA analogs and conjugates thereof which should be useful to those interested in folates as well as receptor targeting in general.

MATERIALS AND METHODS

Materials. Pteroic acid (Pte) and N^{10}-trifluoroacetylpteroic acid were prepared according to Xu *et al.* (8). Peptide synthesis reagents were purchased from NovaBiochem (La Jolla, CA) and Bachem (San Carlos, CA). Folate-free RPMI media

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(FFRPMI) and PBS were obtained from Gibco, Grand Island, NY. Bovine soluble milk folate binding protein (sFBP) was purchased from Scripps (item #F0524). ^{3}H-thymidine was purchased from Moravek Biochemicals, Brea, CA. All other common reagents were purchased from Sigma (St. Louis. MO) or other major suppliers. The test articles EC20, EC119, EC17, EC72, EC140 and EC145 were produced by Endocyte, Inc. (West Lafayette, IN). Their syntheses, purifications and analytical characterizations have been described in detail elsewhere (9–13). Des-glutamyl CB3717 and CB3717 were prepared according to known procedures (14,15). Methotrexate (MTX) was purchased from Sigma, and pemetrexed (PMX) was purchased from a commercial pharmacy as Alimta®.

Synthesis of EC216. In a dry 10 mL round bottom flask, pteroyl-cysteine (Pte-Cys-OH; 7.6 mg, 18.3 μmol) and 3-(4-desacetylvinblastinyl)hydrazinecarboxylic acid 2-pyridyldithioethyl ester (15 mg, 15.3 μM) were dissolved in 0.8 mL of dimethylsulfoxide (DMSO) under argon atmosphere. Diisopropylethylamine (DIPEA; 54 μL, 0.31 mM, 20 equiv.) was added to the above solution, and the resulting clear solution was stirred under argon for 3 h. Progress of the reaction was monitored by analytical HPLC (10 mM ammonium acetate, pH=7.0 and acetonitrile). The reaction mixture was filtered and injected on a prep-HPLC column (Waters XTerra C18, 7 μm, 19×300 mm). Elution with 1 mM sodium phosphate buffer, pH=7.0 (a), and acetonitrile (b) (method: 1% B to 80% B in 30 min at 15 mL/min) yielded pure fractions containing the product. Pure fractions were combined and acetonitrile was removed under reduced pressure at ambient temperature. The resulting EC216 conjugate was isolated after freeze-drying for 48 h (10 mg, 51%). ^{1}H NMR (300 MHz, DMSO-d_6 with D_2O) δ 8.63 (s, 1H), 7.58 (d, J=8.7 Hz, 2H), 7.36 (d, J=8.4 Hz, 1H), 7.24 (d, J=8.1 Hz, 1H), 7.10–6.80 (m, 2H), 6.65 (d, J=9.0 Hz, 2H), 6.41 (s, 1H), 6.19 (s, 1H), 5.68 (m, 1H), 5.56 (d, J=10.2 Hz, 1H), 4.46 (s, 2H), 4.35–3.90 (m, 4H), 3.69 (s, 3H), 3.45–2.20 (m, 23H), 2.05–1.85 (m, 2H), 1.56 (m, 2H), 1.40–0.95 (m, 8H), 0.78 (t, J=7.5 Hz, 3H), 0.71 (t, J=7.2 Hz, 3H); LCMS (ESI): $(M + H)^+$=1286.34.

Synthesis of EC0384. The peptide fragment Pte-γ-Glu-Asp-Arg-Asp-Asp-Cys-OH (EC119; 97 mg, 1 eq.) was dissolved in 5 mL of water under argon while adjusting the pH to 7. To the resulting yellow solution was added a solution of the pyridyldithio-derivative of budesonide (60 mg, 1 eq.) in 5 mL of tetrahydrofuran (THF). The reaction mixture was stirred for 30 min under argon at room temperature. Progress of the reaction was monitored by analytical HPLC (10 mM ammonium acetate, pH=7.0 and acetonitrile). The reaction mixture was filtered and injected on a prep-HPLC column (Waters XTerra C18, 7 μm, 19×300 mm). Elution with 1 mM sodium phosphate buffer, pH=7.0 (a) and acetonitrile (b) (method: 1% B to 50% B in 30 min at 15 mL/min) yielded pure fractions containing the product. Pure fractions were combined and acetonitrile was removed under reduced pressure at ambient temperature. The resulting EC0384 conjugate (68 mg) was isolated as a yellow powder after freeze-drying for 48 h. ESI-MS: $(M + H)^+$=1579.20; ^{1}H NMR (300 MHz, D_2O): δ 0.60 (d, J=5.3 Hz, 3H), 0.67 (t, J=7.3 Hz, 2H), 0.73 (t, J=7.3 Hz, 2H), 0.95 (m, 1H), 1.15 (m, 6H), 1.35 (m, 6H), 1.52 (m, 2H), 1.61(m, 1H), 1.75 (m, 2H), 1.90 (m, 3H), 2.12 (m, 1H), 2.37 (m, 3H), 2.40 (m, 1H), 2.53 (m, 5H), 2.81 (m, 3H), 3.04 (m, 3H), 4.05 (br, 1H), 4.14 (m, 1H), 4.27 (m, 4H), 4.45 (m, 6H), 4.85 (m, 3H), 5.85 (d, J=7.6 Hz, 1H), 6.15 (t, J=8.8 Hz, 1H), 6.52 (t, J=8.2 Hz, 2H), 7.27 (dd, J=3.5, 6.5 Hz, 1H), 7.44 (t, J=8.5 Hz, 2H), 8.58 (d, J=9.7 Hz, 1H); ESI-MS: $(M + H)^+$=1579.20

Cell Culture. KB cells (FR-positive human nasopharyngeal cells, originally obtained from ATCC; ~4×10^6 FR/cell) were grown continuously as a monolayer using FFRPMI containing 10% heat-inactivated fetal calf serum (HIFCS) at 37°C in a 5% CO_2/95% air-humidified atmosphere with no antibiotics. The HIFCS contained its normal complement of endogenous folates which enabled the cells to sustain growth in this more physiologically relevant medium (1). All cell experiments were performed using FFRPMI containing 10% HIFCS (FFRPMI/HIFCS) as the growth medium unless otherwise specified.

Cell-Free Relative Affinity Assay. One hundred microliters of a FBP 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 on ice with 100 μL/well of freshly-prepared PBS-T containing 0.2% gelatin, and then washed three additional times with PBS-T. PBS solutions (100 μL per well) containing 100 nM of ^{3}H-FA in the absence and presence of increasing concentrations of unlabeled FA or EC145 were added to designated wells in triplicate. Plates were incubated at 37°C for 1 h with gentle shaking and then

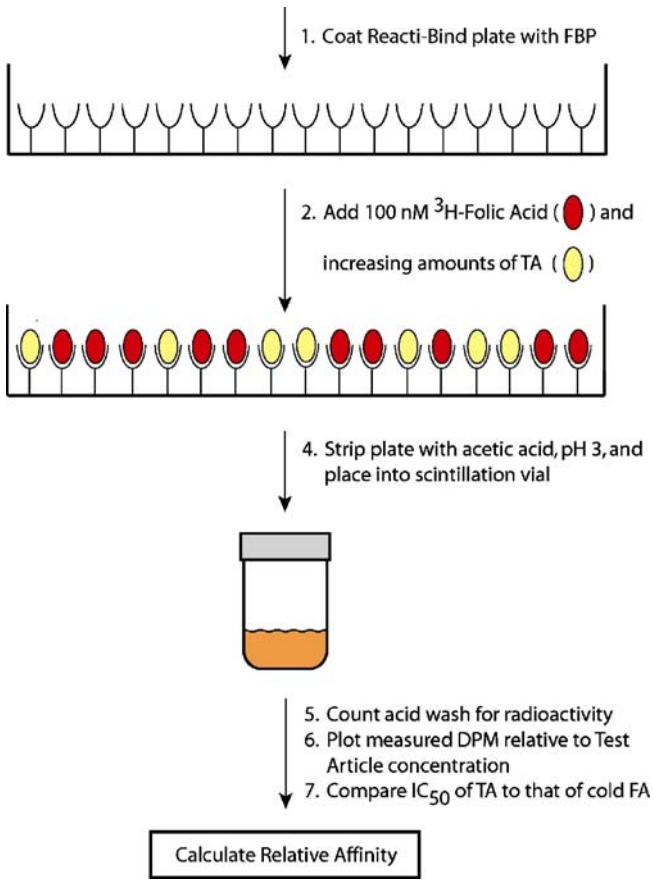
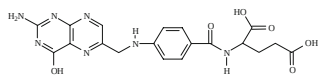
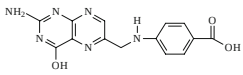
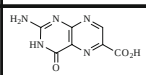
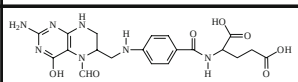
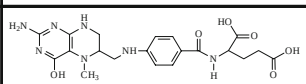
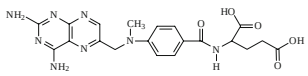
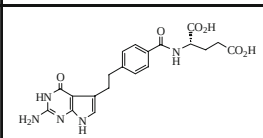
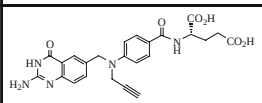
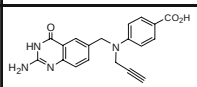
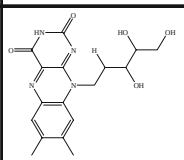


Fig. 1. Schematic design of a cell-free affinity assay. ^{3}H-FA, *red oval*; test article (*TA*), *yellow oval*.

rinsed three times with PBS-T. Wells were stripped 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. Wells exposed to only the 100 nM ^{3}H-FA solution (no competitor) were designated as negative controls, whereas wells exposed to 100 nM ^{3}H-FA plus 1 mM unlabeled FA served as positive controls; DPMs measured in the latter samples (representing non-specific binding of label) were subtracted from the DPM values from all samples. Relative affinities were defined as the inverse molar ratio of compound required to displace 50% of ^{3}H-FA bound to FR on KB cells, and the relative affinity of FA for the FR was set to 1.

Cell-Based Relative Affinity Assay. The relative affinity of each test article was determined based on a method initially described by Westerhoff *et al.* (16) with modifications. Briefly, 100,000 FR-positive KB cells were seeded into each well of 24-well Falcon plates and allowed to form adherent monolayers (>75% confluent) overnight in FFRPMI/HIFCS. Typically, no more than five plates (120 test wells) were used for any given experiment. Spent incubation medium was replaced with FFRPMI (with a specified amount of HIFCS) and containing 100 nM of ^{3}H-FA in the absence and presence of increasing concentrations of unlabeled FA or EC145. Cells were incubated for 1 h (in triplicate wells) on ice or in an incubator at 37°C (as specified in the figure legends) and then rinsed 3 times with 0.5 mL of PBS. Five hundred microliters of 1% sodium dodecylsulfate in PBS were added to each well; after 5 min, cell lysates were collected, transferred to individual vials containing 5 mL of scintillation cocktail, and then counted for radioactivity. Cells exposed to only the ^{3}H-FA in FFRPMI (no competitor) were designated as negative controls, whereas cells exposed to the ^{3}H-FA plus 1 mM unlabeled folic acid served as positive controls; DPMs measured in the latter samples (representing non-specific binding of label) were subtracted from the DPM values from all samples. Importantly, both

Table I. Relative Affinities of Vitamers and Derivatives for the Folate Receptor

Test Article	Class	Structure	Relative Binding Affinity	Fold Weaker Affinity from FA
Folic Acid (Pte-Glu)	Vitamer		1.000	1
Pteroic Acid (Pte)	Folate precursor		0.011	91
Pterin-6-carboxylate	Pterin analog		0.000	n/a
5-formyl-tetrahydrofolate (Leucovorin)	Vitamer		0.008	125
5-methyl-tetrahydrofolate (5MTHF)	Vitamer		0.070	14
Methotrexate (MTX)	Anti-folate		0.018	56
Pemetrexed (PMX)	Anti-folate		0.002	500
CB3717	Anti-folate		1.000	1
Des-glutamyl CB3717	Anti-folate precursor		0.002	500
Riboflavin	Non-folate vitamer		0.000	n/a

positive and negative controls were tested and applied for each set of plates. Relative affinities were defined as described above.

RESULTS

Assay Format. The *in vitro* RA assay described within measures a ligand's ability to directly compete with FA for binding to FRs. To date, cultured cells have most commonly been used as the FR source (9,11,12,16). However, we have also evaluated the use of a cell-free, immobilized FR plate system for this assay. As diagramed in Fig. 1, this latter method utilized a microtiter plate to which commercially-available bovine milk soluble FBP had been chemically anchored. Similar to the cell-based method, ^{3}H-FA can compete directly with increasing concentrations of test article for binding to the attached FRs. For either assay, a relative affinity value of 1.0 implies that the test article ligand has an affinity equal to that of FA for the FR. Likewise, values lower than unity reflect weaker affinity, and values higher than unity reflect stronger affinity.

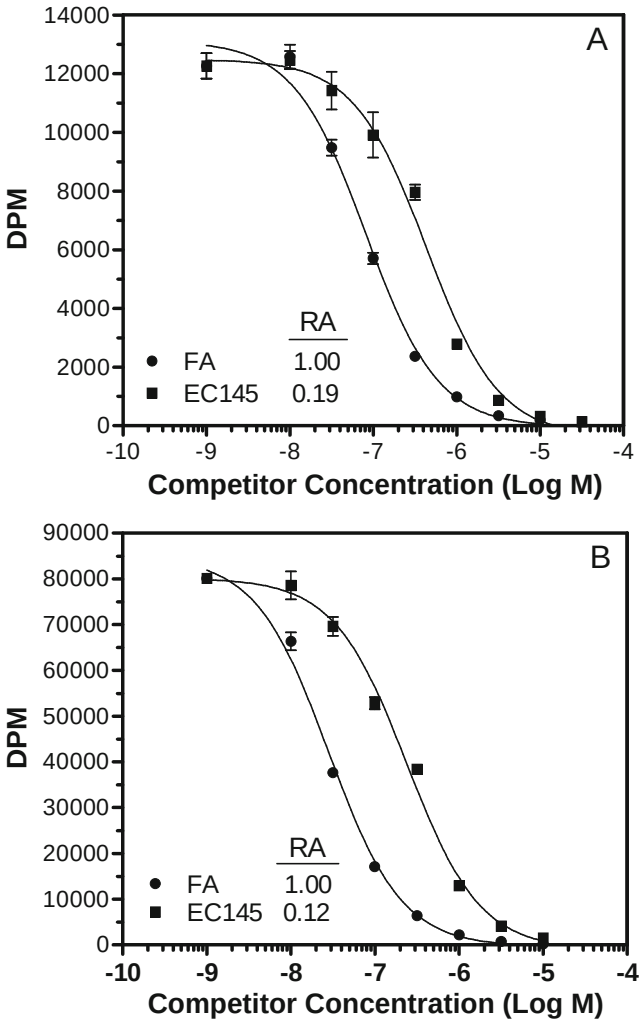


Fig. 2. Comparison of cell-free and cell-based affinity assays. **a** KB cell-based relative affinity assay. **b** Cell-free relative affinity assay. Both assays were performed in the absence of serum while the plates were sitting on a bed of ice. FA, (*circle*). EC145, (*square*). Data represent the average ±1 SD (n=3).

The functionalities of both the cell- and cell-free methods were directly compared using EC145, a folate conjugate of the microtubule destabilizing agent, desacetylvinblastine monohydrazide [DAVLBH; see Table I and (13,17)]. When evaluated under ice-cold incubation conditions, EC145 was experimentally determined to have an RA of 0.19 relative to that of FA for human FRs (KB cells) and an RA of 0.12 for the plate-anchored bovine milk sFBP (see Fig. 2). Indeed, the results between the two assays are similar (less than a factor of 2). However, considering that (1) the KB cell assay involves the testing with the membrane-bound, human form of the FR, (2) the cell-free assay uses non-human (bovine) soluble folate binding protein, (3) the cell-free microplate-based assay is more expensive to conduct, and (4) the cell-based assay requires less steps to complete, we opted to use the cell-based assay for all future experiments.

Temperature-Dependent Effects. Early into our investigation, we examined the effect of incubation temperature on the relative affinities for a few common pteroates and antifolates. As shown in Fig. 3a, Pte was experimentally found to have an RA of 0.42, or an affinity 2.4-fold weaker than FA when tested using cells that were incubated on ice. This value was surprisingly high because other published reports had indicated that Pte displayed extremely low affinity for the FR (18,19). We wondered if the discrepancy between our data and the historical data was due to the applied experimental temperatures. Interestingly, upon repeating this assay at 37°C, Pte's RA decreased dramatically to 0.01, or an affinity 100-fold weaker than FA. A similar bizarre pattern was also noted for leucovorin (LV; also called 5-formyltetrahydrofolate or folinic acid; see panels c and d), and for the antifolate, PMX (see panels e and f). Interestingly, regardless of the experimental temperature, the antifolate MTX was found to be a poor binder to the FR (see panel e and Table I). Such temperature-dependent effects on affinity were not observed when FA-drug conjugates were tested under similar conditions. In fact as shown in Fig. 4, the RAs of both EC72 [a mitomycin C conjugate of FA; (11)] and EC17 [a fluorescein conjugate of FA; (10)] somewhat increased with temperature. Because our laboratory is obviously interested in studying the biochemical effects of FA-drug conjugates under physiological conditions, we chose to fix the temperature variable at 37°C for all future studies.

Serum-Dependent Effects. All of the aforementioned studies were conducted in the absence of serum. Yet, it is routine practice to evaluate the activity of folates, FA-drug conjugates, and even antifolates with cells in the presence of serum. To assess the impact that serum could have on the RA assay, we measured the cell-associated level of ^{3}H-FA under conditions where the percent serum in the medium was varied. As shown in Fig. 5a, ^{3}H-FA binding to KB cells was not found to be affected by fetal bovine serum, even when present at 100%. Likewise, increasing the percent sera from 0% to 100% had only a minor effect on unlabeled FA's ability to compete with ^{3}H-FA for binding to KB cells (see Fig. 5b), since the calculated RA values for FA varied by only 13% among all tested conditions. Interestingly, the RA value for the desacetylvinblastine monohydrazide FA conjugate, EC145, was found to remain nearly constant at 0.48 (±0.02) when sera was present at concentrations ≥10%; but, a 2.4-fold decrease in RA was reproducibly observed for EC145

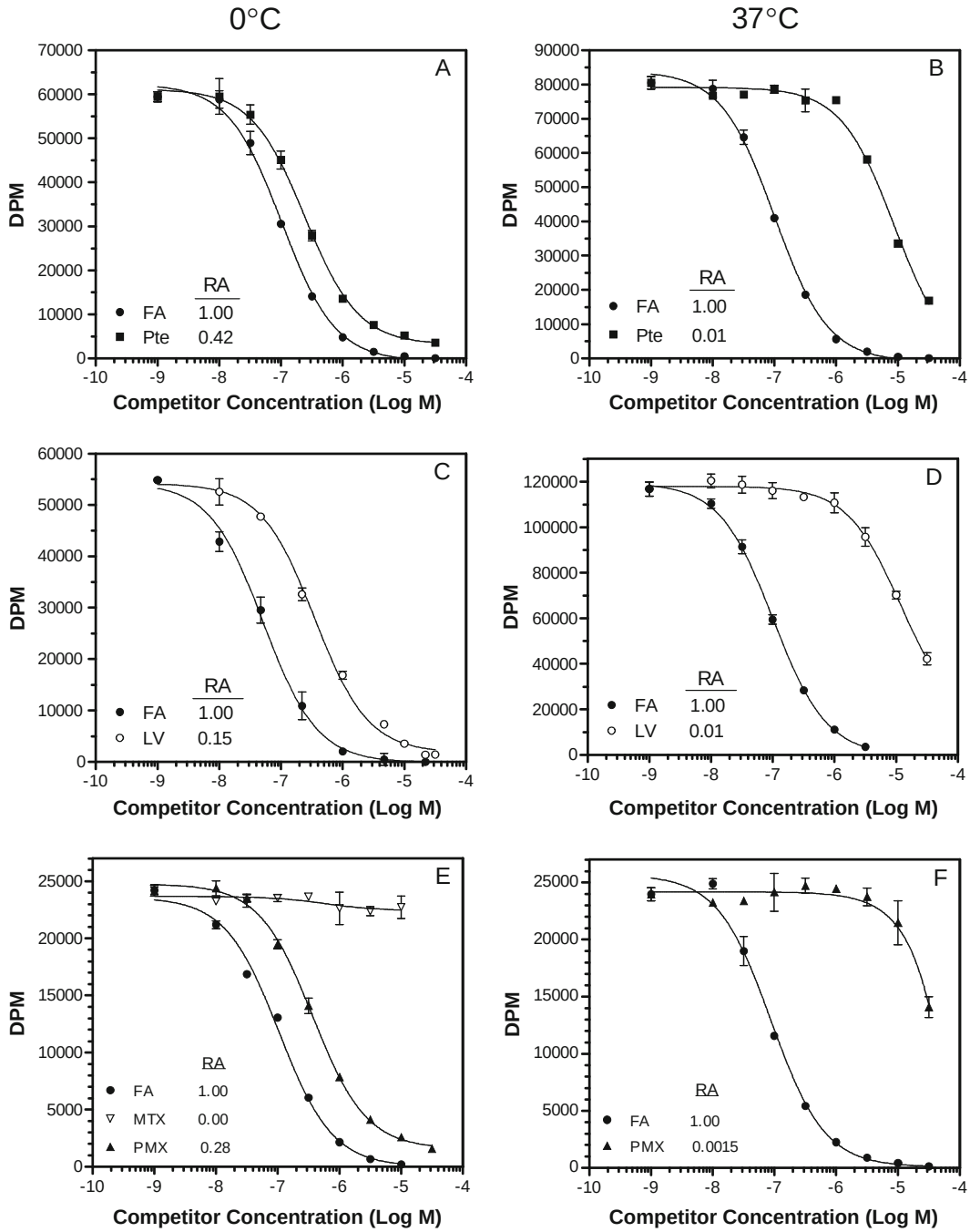


Fig. 3. Effect of incubation temperature on the relative affinity of pteroic acid, leucovorin, MTX and PMX. **a** Pte acid on ice. **b** Pte at 37°C. **c** LV on ice. **d** LV at 37°C. **e** MTX and PMX on ice. **f** PMX at 37°C. Each assay was conducted using adherent KB cells as the FR source without serum in the test medium. FA, or Pte-γGlu, (*filled circle*); LV, (*empty circle*); Pte, (*square*); MTX, (*inverted triangle*); PMX, (*triangle*). Data represent the average ±1 SD (n=3).

when evaluated in the absence of serum (see Fig. 5c). To confirm this latter finding, the RA of a related *Vinca* alkaloid conjugate called EC140 (see structure in Table I) was tested under similar conditions. As shown in Fig. 5d, the addition of 10% serum increased the RA of EC140 4-fold, from 0.12 (no serum) to 0.50. Notably, both EC145 and EC140 remain stable in 95% serum for incubation periods longer than 2 h (via HPLC-UV; data not shown); so, degradation and release of free FA from these conjugates is not responsible for these observations. Although the mechanism by which serum improves a FA-drug's RA is currently unknown, it is possible (but speculative) that dynamic interactions of the lipophilic drug payloads with serum protein somehow aids in the presentation of the FA moiety to the binding pocket of the FR. Regardless, a test medium which includes 10% fetal bovine serum was chosen as our standard solution for all future RA analyses.

Relative Affinity of Folate Analogs. Employing our defined assay conditions, which included the use of adherent KB cells as the FR source and a 10% serum-supplemented

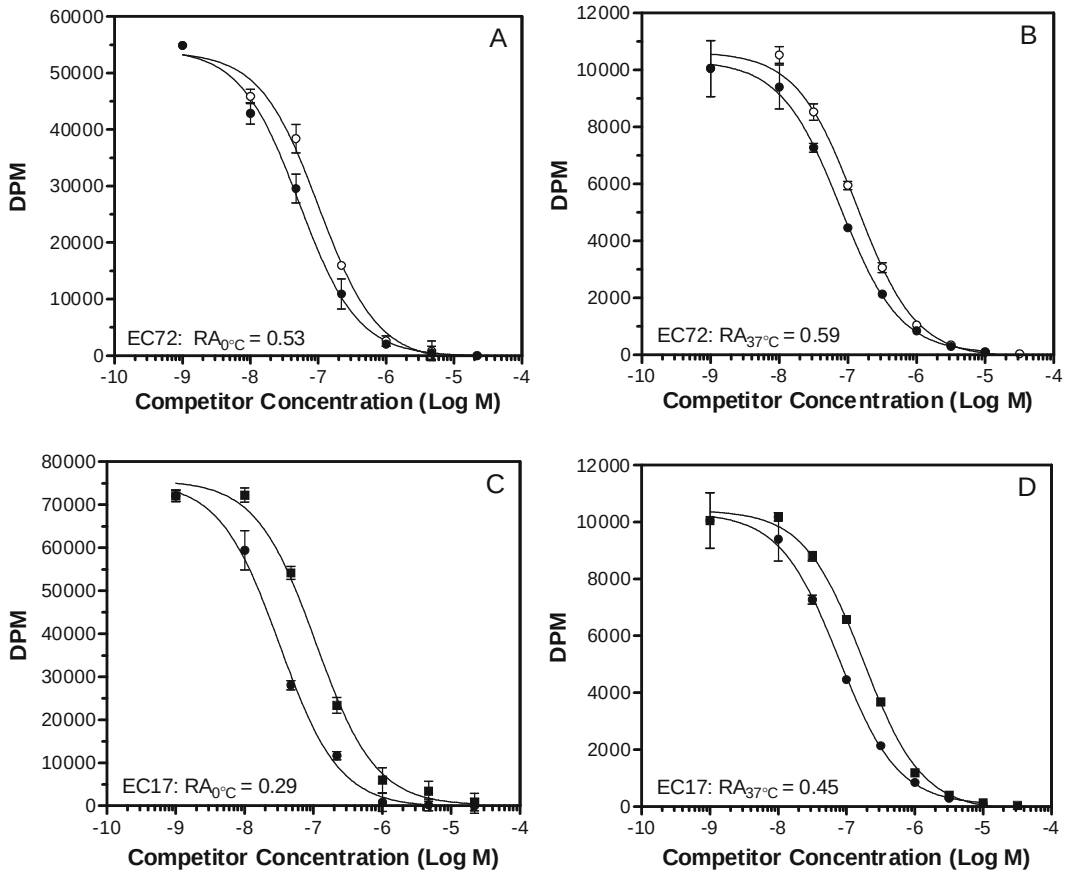


Fig. 4. Effect of incubation temperature on the relative affinity of Pte-γGlu-drug conjugates. **a** EC72 on ice. **b** EC72 at 37°C. **c** EC17 on ice. **d** EC17 at 37°C. Each assay was conducted using adherent KB cells as the FR source without serum in the test medium. FA, or Pte-γGlu, (*filled circle*); EC72, (*empty circle*); EC17 (*square*). Data represent the average ±1 SD (n=3).

medium, a series of folate analogs were evaluated for their ability to compete for binding with ^{3}H-FA at 37°C. As shown in Table I, removal of the glutamyl (Glu) residue in FA to produce Pte had caused a 91-fold reduction in RA (refer to Fig. 3b). Such results are consistent with other literature reports (18,19). Further removal of the *p*-aminobenzoyl moiety from Pte was found to completely eliminate competitive binding capability, since pterin-6-carboxyllic acid failed to block ^{3}H-FA's binding to KB cells at all concentrations evaluated. The most prevalent and natural serum-derived folate, 5-methyltetrahydrofolate (20), and its 5-formyl counterpart, leucovorin, were found to display 14- and 125-fold weaker affinities for the FR, respectively; likewise, the popular anti-folates MTX and PMX showed a 56- and 500-fold weaker affinity, respectively. Another well-known antifolate, CB3717, was found to have an RA value equivalent to FA. Although this property has previously been reported for this compound (16), we were surprised to find that its des-glutamyl counterpart had an RA value ~500-fold lower (i.e. a much more dramatic RA decrease compared to the FA/Pte paired results; see Table I). These data collectively suggest that the glutamyl moiety, or at least a hydrophilic moiety (21), is required to be positioned in close proximity to the p-aminobenzoyl (or aryl) group of the pteroate to afford optimal binding to the FR. Finally, since it has been reported that riboflavin binding protein and FR share ~27% homology (22), we elected to test riboflavin's RA to the KB-derived FR. But as shown in Table I, even a 300-fold molar excess of this vitamin was not able to compete with ^{3}H-FA's binding to KB cells.

Influence of Drug Cargo on Relative Binding Affinity. The focus of our laboratory is aimed towards the production of FA conjugates of drugs that are useful for applications in radiodiagnostic imaging, chemotherapy, immunotherapy and inflammation. We have previously reported RA values for a few conjugates, including three of our current clinical candidates (9,11,12,17); however, the assay conditions in those reports were not standardized. As shown in Table II, we summarize the RA values for some previously-reported conjugates as well as many new ones that were all tested under identical assay conditions (namely, the use of adherent KB cells as the FR source, and a 10%-serum-supplemented 37°C medium). The drug "payloads" for these conjugates widely varied from a metal-chelating peptide, to potent lipophilic chemotherapeutics. Yet, for the most part the RA values all fell within a factor of ~2. We believe this indicates that a flexible, hydrophilic molecular spacer placed in-between the FA and drug moieties of the conjugates generally allows for efficient binding of the FA moiety to the FR. This hypothesis is somewhat supported by our findings that a shorter spacer had a negative impact on the RA value for the desacetylvinblastine monohydrazide conjugate, EC216 (com-

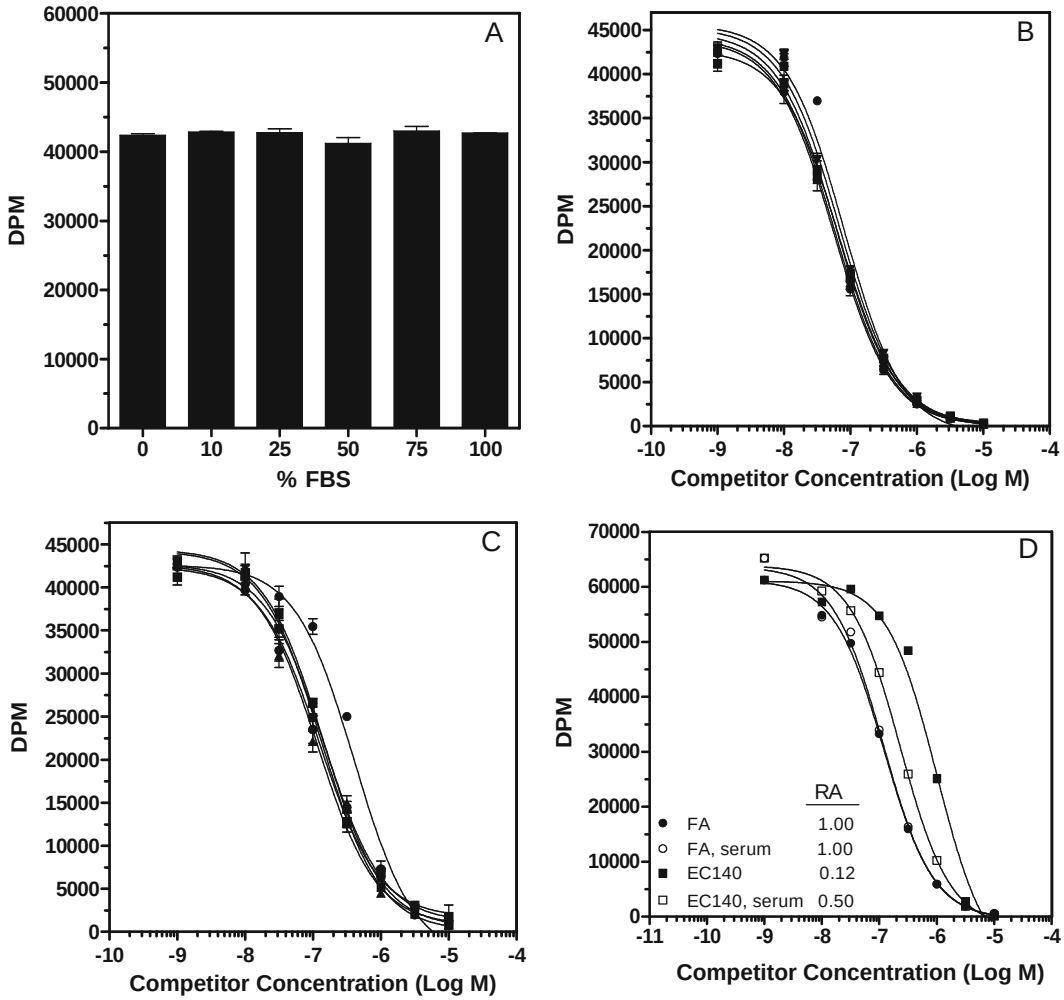


Fig. 5. Serum-dependent effects on relative affinity. **a** Binding of FA to KB cells in the presence of increasing amounts of fetal bovines serum. **b**, **c** RA of FA and EC145, respectively [percent serum: 0% (*filled circle*), 10% (*filled square*), 25% (*triangle*), 50% (*open square*), 75% (*inverted triangle*), 100% (*empty circle*). **d** Effect of 0% or 10% serum on the RA of EC140. Data represent the average ±1 SD (n=3).

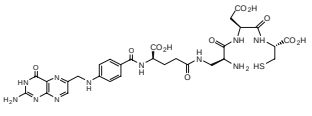
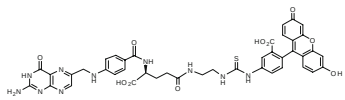
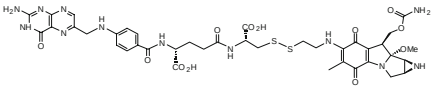
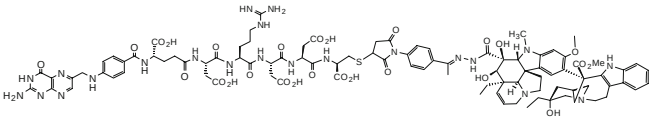
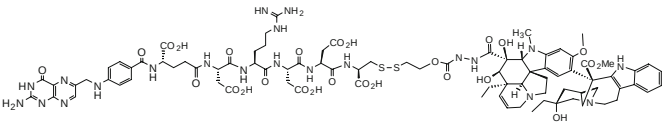
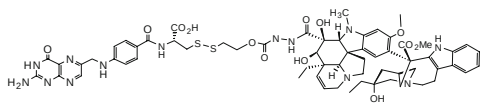
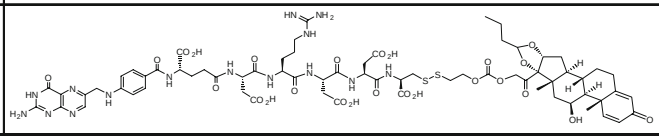
pare to EC145 in Table II). However, the spacer is likely not the only determinant that can affect a conjugate's RA, because we recently produced a water-soluble steroid conjugate with a poor 20-fold weaker binding affinity (EC0384; Table II). While it is interesting to speculate about the possible reason for EC0384's lower RA (see "DISCUSSION"), it should be mentioned that this latter result is rather unusual. But overall, the vast majority of our 600+ FA-drug conjugates made and tested to date do display RA values that are within a factor of ~3 from FA (C.P. Leamon, personal observations).

DISCUSSION

Over the course of developing FA-targeted therapeutics, we carefully evaluated our options for an assay that could reproducibly determine a conjugate's affinity for the FR protein. It was obvious that direct binding measurements using radiolabeled test articles was neither practical nor economical, and the lack of a suitable source for purified, human FR made Biacore-based methods less attractive to us. Instead, we adopted and modified a "relative affinity" method reported previously where the affinity of an unlabeled test article could be assessed in a competitive binding format (16).

When we first began investigating the effects of FA-drug conjugation on overall FR affinity, we had used suspended FR-positive KB cells that were held at cold temperatures (typically on ice) for brief periods of time in serum-free medium (11). Useful data were collected, especially in regards to comparing the impact of changing the drug and linker moieties within our conjugates. However, we quickly noticed an anomaly: the base pteroate molecule, Pte, was reproducibly found to compete rather well with ^{3}H-FA (Pte-Glu) for binding to the human FR (see Fig. 3a). We were genuinely skeptical about these results, for previous literature reports suggested that Pte was a poor binder to the FR (18,19). But upon further examination, it was clear that the published affinity data with Pte were not collected using assays performed at low temperatures. Upon repeating our assay at 37°C (Fig. 3b), we were pleased to observe that Pte did in fact not compete very well for FR binding. Interestingly, this temperature-dependent affinity pattern was also observed with leucovorin (Fig. 3c, d) and PMX (Fig. 3d, f) but not with

Table II. Relative Affinities of Folic Acid Drug Conjugates for the Folate Receptor

Test Article	Class	Structure	Relative Binding Affinity	Fold Weaker Affinity from FA
EC20 (Pte-γGlu–βDpr-Asp-Cys)	Folate-based Imaging Agent		0.920	1.1
EC17 (Pte-γGlu-eda-FITC)	Folate-fluorophore Agent		0.840	1.2
EC72 (Pte-γGlu-Cys-ss-MMC)	Folate-targeted chemotherapeutic		0.650	1.5
EC140 (EC119-Hyd-DAVLBH)	Folate-targeted chemotherapeutic		0.500	2.0
EC145 (EC119-ss-DAVLBH)	Folate-targeted chemotherapeutic		0.470	2.1
EC216 (Pte-Cys-ss-DAVLBH)	Folate-targeted chemotherapeutic		0.046	21.7
EC0384 (EC119-ss-budesonide)	Folate-steroid		0.050	20.0

the known high affinity ligand, FA. The mechanism responsible for this apparent temperature-dependent weakening of ligand affinity is not yet understood. However, it should be noted that KB cells also express functional levels of another folate/antagonist transporter, called the reduced folate carrier (RFC), which is known to transport leucovorin, PMX and other monocarboxylic anions inside cells (23). Thus, under our defined experimental conditions, it is possible that a major portion of these competitors could have been taken up intracellularly via the RFC at 37°C, resulting in much lower extracellular concentrations available for competition with ^{3}H-FA for binding to the FR.

The presence of serum in the test medium also proved to be important to FA-drug conjugates, but not to FA itself. Thus, the RA values for both *Vinca* conjugates, EC145 and EC140, were shown to dramatically increase in the presence of serum (2.4- and 4.1-fold, respectively). These results could not be explained by release of FA from the conjugates because both agents were subsequently found to remain stable, even in 95% serum, for more than 2 h. Therefore, some unexplained protein-assisted mechanism is thought to be responsible for improving the presentation of FA-drug conjugates to cell surface FRs.

Using a standardized protocol which included the use of adherent FR-positive KB cells exposed to 100 nM ^{3}H-FA in the presence of increasing concentrations of competitor in 10% serum-supplemented medium at 37°C, we screened a wide variety of folate analogs and FA-drug conjugates and determined their RA values for comparison (see Tables I and II). Among the folate analogs, it was interesting to confirm the importance of having a charged moiety in close proximity to the Pte group. For example, the RA value increased 90 to ~500-fold when a Glu residue was attached to Pte and N^{10}-propargyl 5,8-dideazapteroic acid, respectively. Notably, although is it more "natural" to place a Glu residue at this position, it is known that other amino acids can substitute for Glu without dramatically compromising binding affinity (21,24,25).

For the series of FA-drug conjugates examined here, it was also interesting to find that RA values remained within a factor of ~2 despite the wide structural variances present among the different drug payloads. But, two exceptions were presented. The first showed that shortening the peptide-based spacer of EC145 from γGlu-Asp-Arg-Asp-Asp-Cys down to only a Cys residue (in EC216) caused a 10-fold decrease in RA. Admittedly, eliminating many of the charged groups in the "spacer" region likely compromised the total water solubility characteristics of the conjugate, but EC216 was observed to remain in solution (even at 30 μM) throughout the assay period. Therefore, the lower RA value observed for

EC216 probably resulted from some sterical interference caused by its drug's (DAVLBH) closer proximity to the Pte unit. The second exception we presented was that related to the 20-fold lower RA value which resulted when a lipophilic steroid had been anchored to folate (EC0384; see Table II). Although a more detailed investigation is necessary to fully understand why the steroid interfered with binding, we have since determined that EC0384's serum protein binding is >96%. Since the majority of folate conjugates bind serum protein less than 80% (C.P. Leamon, personal observations), this factor could be responsible, in part, for the lower observed RA.

Overall, RA information is valuable to have during the course of developing a targeted agent because it aids in the design of more effective and sometimes predictable preclinical leads. We utilize this assay when evaluating every new FA-drug conjugate prepared in our laboratory (>700 to date), and we have found that it is more common to observe a slight reduction in RA rather than a large one. There are likely many mechanisms by which a drug negatively affects a conjugate's affinity, and the methodology described above enables the researcher to quickly determine if a problem exists. If so, such information should trigger additional studies to define the molecular basis of that problem and to identify possible solutions that can restore a conjugate's affinity.

ACKNOWLEDGEMENTS

The authors wish to thank Marilynn Vetzel for providing the numerous plates of adherent KB cells throughout this investigation.

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