Objective. To determine the expression of folate receptor β (FRβ) in synovial biopsy tissues and peripheral blood lymphocytes from rheumatoid arthritis (RA) patients and to identify novel folate antagonists that are more selective in the targeting and internalization of FRβ than methotrexate (MTX).

Methods. Immunohistochemistry and computer-assisted digital imaging analyses were used for the detection of FRβ protein expression on immunocompetent cells in synovial biopsy samples from RA patients with active disease and in noninflammatory control synovial tissues. FRβ messenger RNA (mRNA) levels were determined by reverse transcription–polymerase chain reaction analysis. Binding affinities of FRβ for folate antagonists were assessed by competition experiments for 3H-folic acid binding on FRβ-transfected cells. Efficacy of FRβ-mediated internalization of folate antagonists was evaluated by assessment of antiproliferative effects against FRβ-transfected cells.

Results. Immunohistochemical staining of RA synovial tissue showed high expression of FRβ on macrophages in the intimal lining layer and synovial sublining, whereas no staining was observed in T cell areas or in control synovial tissue. Consistently, FRβ mRNA levels were highest in synovial tissue extracts and RA monocyte-derived macrophages, but low in peripheral blood T cells and monocytes. Screening of 10 new-generation folate antagonists revealed 4 compounds for which FRβ had a high binding affinity (20–77-fold higher than for MTX). One of these, the thymidylate synthase inhibitor BCG 945, displayed selective targeting against FRβ-transfected cells.

Conclusion. Abundant FRβ expression on activated macrophages in synovial tissue from RA patients deserves further exploration for selective therapeutic interventions with high-affinity–binding folate antagonists, of which BCG 945 may be a prototypical representative.

The folate antagonist methotrexate (MTX) is the most widely applied disease-modifying antirheumatic drug (DMARD) in the treatment of patients with rheumatoid arthritis (RA) (1). It is used either as single agent or in combination with other DMARDs (e.g., sulfasalazine and hydroxychloroquine), and MTX use is obligatory in most treatment strategies involving biologic agents (anti–tumor necrosis factor α [anti-TNFα] or anti-CD20 monoclonal antibodies) (2–5).

The first pivotal step in the cellular pharmacology of MTX is its entry into the cell, which can be mediated by at least 3 different routes: the reduced folate carrier (RFC) (6,7), membrane-associated folate receptors
ceptor (8). Interestingly, selective expression of the folate re-
take by FR (11) and animal models of arthritis (12,13) has been
synovial fluid from the inflamed joints of RA patients
specific types of cancer (e.g., ovarian cancer), while the
identified. The isoform of MFR (16,17). Three isoforms of MFR (α, β, and γ) have been
for imaging of arthritis and, therapeutically, for selective antibody–guided or folate conjugate–
guided delivery of toxins and other small molecules/ macromolecules (13–15). Thus far, targeting of FR with 
folate antagonists has only been explored in cancer cells/tissues that overexpress FRα (16,17).

Over the last 2 decades, a second generation of 
folate antagonists has been designed and evaluated clinically from the perspective of circumventing common mechanisms of resistance to MTX, including impaired transport via the RFC, defective polyglutamation, increased activity of the target enzyme dihydrofolate reductase (DHFR), and/or enhanced drug efflux (7). Based on this background, second-generation antifolates include compounds that are more efficiently transported via the RFC, are more efficiently polyglutamylated, or are independent of polyglutamation, or they target key enzymes other than DHFR in folate metabolism, such as thymidylate synthase (TS) or glycaminide ribonucleotide transformylase (GART) (6,18,19). In the present study, we investigated whether distinct second-generation antifolates may serve as selective targeting drugs for FRβ-expressing cells in synovial tissue and/or immunocompetent cells from patients with RA. We identified the TS inhibitor BCG 945 as a prototypical antifolate drug that fulfilled the criteria of a high binding affinity for FRβ and a low affinity for the RFC, thereby enabling selective uptake by FRβ-expressing cells.

PATIENTS AND METHODS

Drugs. Folic acid was obtained from Sigma (St. Louis, MO), and l-leucovorin was purchased from Merck Eprova (Schaaffhausen, Switzerland). MTX was a kind gift of Pharmachemie (Haarlem, The Netherlands). Pemetrexed (Altilma; Eli Lilly, Indianapolis, IN) (20) was obtained via the VU University Medical Center pharmacy department. The following folate antagonist drugs were also obtained: raltitrexed (Tomudex/ZD1694; AstraZeneca, London, UK) (21), PT523 and PT644 (Dr. A. Rosowsky, Harvard Medical School, Boston, MA) (22,23), GW1843 (Gluco Wellcome, Research Triangle Park, NC) (24), CB300635 (Institute of Cancer Research, Sutton, UK) (25), plevitrexed (BGC 9331; BTG International, London, UK) (26), BGC 945 (both the 6R,S and 6S forms; BTG International) (27), 5,10-dideazatetrahydrofolate (DDATHF; Eli Lilly) (28), and AG2034 (Agouron/Pfizer Pharmaceuticals, San Diego, CA) (29). (Illustrations of the chemical structures of these folate antagonists are available online at http://jwvanderheijden.blogspot.com/) Preclinical and clinical background information on these antifolates has previously been published (6,18). 3′,5′,7,9-3H-folic acid (20–40 Ci/m mole; MT783) was purchased from Moravek (Brea, CA).

Cell lines. Wild-type (WT) Chinese hamster ovary (CHO) cells, CHO cells transfected with FRβ (CHO/FRβ) (30), and human nasopharyngeal epidermoid KB cells expressing FRα (17) (all from American Type Culture Collection [ATCC], Manassas, VA) were grown in folic acid–free RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.15 mg/ml of proline, and 100 units/ml penicillin and streptomycin. The 3H-folic acid binding capacities of CHO/FRβ and KB cells were 0.5–1 pmoles/10⁶ cells and 20–40 pmoles/10⁶ cells, respectively. Human monocytic macrophage THP-1 cells (ATCC) were grown in RPMI 1640 medium containing 2.2 μM folic acid supplemented with 10% FCS, 2 mM L-glutamine, 0.15 mg/ml of proline, and 100 units/ml of penicillin and streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Synovial tissue samples. In this study, we analyzed synovial tissue biopsy samples from the knee joints of 15 RA patients with active disease obtained before and after 4 months of treatment with MTX, including impaired transport via the RFC, defective polyglutamation, increased activity of the target enzyme dihydrofolate reductase (DHFR), and/or enhanced drug efflux (7). Based on this background, second-generation antifolates include compounds that are more efficiently transported via the RFC, are more efficiently polyglutamylated, or are independent of polyglutamation, or they target key enzymes other than DHFR in folate metabolism, such as thymidylate synthase (TS) or glycaminide ribonucleotide transformylase (GART) (6,18,19). In the present study, we investigated whether distinct second-generation antifolates may serve as selective targeting drugs for FRβ-expressing cells in synovial tissue and/or immunocompetent cells from patients with RA. We identified the TS inhibitor BCG 945 as a prototypical antifolate drug that fulfilled the criteria of a high binding affinity for FRβ and a low affinity for the RFC, thereby enabling selective uptake by FRβ-expressing cells.

The arthroscopy procedure was performed as described previously (32) as part of a joint study that was approved by the medical ethics committees of Leiden University Medical Center (Leiden, The Netherlands) and Leeds University Medical Center (Leeds, UK) (33). As noninflammatory control synovial tissue, samples from 7 patients with mechanical joint injury (kindly provided by Dr. B. J. van
Royen, Department of Orthopedic Surgery, VU University Medical Center, Amsterdam, The Netherlands) were used. Before peripheral blood sample collection, all patients signed an informed consent form, and the study on DMARD resistance was approved by the Medical Ethics Committee of the VU University Medical Center, Amsterdam, The Netherlands.

**FRβ immunohistochemistry.** Immunohistochemical staining of cryostat sections (5 μm) of synovial tissue biopsy samples from RA patients and controls was performed using a 3-step immunoperoxidase method as described previously (34,35). Sections were stained with an FRβ-specific antibody (30) (1:3,000 dilution) and with normal rabbit serum isotype control. Macrophages and T cells were stained with 3A5 (1:100 dilution) (36) and phycoerythrin-labeled anti-CD3 (1:25 dilution; Dako, Glostrup, Denmark) monoclonal antibodies, respectively, and with mouse immunoglobulin isotype control. Biotinylated swine anti-rabbit IgG (1:200 dilution; Dako) and rabbit anti-mouse IgG (1:300 dilution; Dako) were used as secondary antibodies. Color was developed with 0.4 mg/ml of aminothylcarbazole. After counterstaining with hematoxylin, sections were mounted on slides.

Stained sections were analyzed for FRβ, 3A5, and CD3 expression by digital image analysis, as described previously (37). Briefly, for each marker, representative regions were used for image acquisition at 400× magnification. These regions were divided into 6 high-power fields with a 3-pixel overlap. Positive cells were evaluated by analyzing 18 consecutive high-power fields and then scoring the numbers of positive cells (per mm²) in the intimal lining layer and the synovial sublining.

**Double-labeling immunofluorescence.** FRβ was detected by horseradish peroxidase–labeled swine anti-rabbit antibodies (1:200 dilution; Dako) and development was with rhodamine/tyramine (1:1,000 dilution; red fluorescence) according to the instructions of the manufacturer (38). CD68 was detected by goat anti-mouse biotinylated antibodies (1:100 dilution; Dako), using streptavidin–Alexa 488 as a substrate (1:750 dilution; green fluorescence) (Molecular Probes, Eugene, OR). Slides were mounted with Vectashield, containing 1 μg/ml 4',6-diamidino-2-phenylindole (for staining of nuclei) (Vector, Burlingame, CA). Cells were examined using a Leica DMRB fluorescence microscope (Leica, Rijswijk, The Netherlands).

**Isolation and culture of peripheral blood cells from RA patients.** Peripheral blood mononuclear cells were isolated from freshly obtained blood samples by gradient centrifugation (35 minutes at 400g) on Ficoll-Paque Plus (Amersham Pharmacia Biotechnologies, Little Chalfont, UK). After centrifugation, the interphase was carefully collected and washed 3 times using phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin. The lymphocyte fraction was counted and resuspended in Iscove’s modified Dulbecco’s medium (Invitrogen, Breda, The Netherlands) containing 10% FCS, 2 mM L-glutamine and 100 μg/ml of penicillin and streptomycin. Monocytes were isolated by adherence after 2 hours of incubation at 37°C in culture flasks, followed by RNA extraction or macrophage differentiation by culturing the monocytes for 7 days in the presence of 10 ng/ml of macrophage colony-stimulating factor (Strathmann Biotec, Hamburg, Germany).

Peripheral blood lymphocytes (PBLs) remaining in suspension after monocyte adherence were collected and used for RNA isolation or were used for T cell activation by incubating them at a density of 1 × 10⁶ cells/ml with monoclonal anti-CD28 (5 μg/ml, CLB-CD28/1; Sanquin, Amsterdam, The Netherlands) and anti-CD3 (1 μg/ml, CLB-T3/4E; Sanquin) in goat anti-mouse immunoglobulin–coated 24-well plates. After 48 hours of stimulation, activated T cells were harvested for RNA isolation, and the activation status was determined by measuring CD25 expression by flow cytometry using a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

**FRβ mRNA expression in synovial tissue and peripheral blood cells from RA patients.** RNA from synovial tissue (n = 7), PBLs (n = 9), monocytes (n = 9), macrophages (n = 25), and activated T cells (n = 22) derived from RA patients was isolated using the Qiagen RNeasy Plus isolation kit (Qiagen, Venlo, The Netherlands) according to the instructions provided by the manufacturer. Frozen synovial tissue samples were powdered by grinding them in a liquid nitrogen–prechilled mortar, RPE buffer (Qiagen) was added, and RNA was isolated. Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Real-time reverse transcription–polymerase chain reaction (RT-PCR) methodology previously described by Qi and Ratnam (39) was used to simultaneously measure levels of mRNA for FRβ and GAPDH (reference gene).

The reverse transcription step was performed using TaqMan Reverse Transcript Reagents from Applied Biosystems (Foster City, CA), following the protocol of the manufacturer. Briefly, 400 ng of total RNA was mixed with random hexamer primers (50 μmoles/liter), RNase inhibitor (1 unit/μl), MultiScribe reverse transcriptase (5 units/μl), and a mixture of dNTPs (2.5 μmoles/liter each) in reverse transcriptase buffer. The 10-μl reaction mixture was first incubated at 25°C for 10 minutes, then at 48°C for 30 minutes, and finally, at 95°C for 5 minutes. The subsequent real-time PCR step for FRβ was performed in the presence of 12.5 μl of PCR MasterMix (Applied Biosystems), 0.5 μl each of forward primer (CTG-GCTCCCTGGCTGAGTTC) and reverse primer (GCC-CAAGGCCTGTATATCCA), and 0.5 μl of TaqMan probe (6-FAM-TCCCTCCAGACTCTGGCCTCACG-TAMRA). The primers and the TaqMan probe for control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were 2 minutes at 50°C, then 10 minutes at 95°C, followed by 40 cycles of 15 seconds each at 95°C, and finally, 1 minute at 60°C. The fluorescence data that were generated were monitored and recorded by the GeneAmp 5700 sequence detection system (Applied Biosystems). All samples were set up in triplicate and normalized to GAPDH values.

**Analysis of the binding affinity of FRβ/FRα and the RFC for new-generation folate antagonists.** An intact cell–binding assay for competitive binding of 3H-folic acid and novel antifolate drugs against FRβ and FRα was performed essentially as described previously (16,40). Briefly, CHO/FRβ cells and KB cells expressing FRα were detached by incubation in PBS plus 1 mM EDTA. Detached cells were suspended in ice-cold HEPES buffered saline (140 mM NaCl, 20 mM HEPES, 6 mM KCl, 2 mM MgCl₂, 6 mM Na₂HPO₄, pH 7.4, with NaOH) to a concentration of 3 × 10⁶ cells/ml and 1 × 10⁶ cells/ml, respectively. One milliliter of cell suspension was added to a series of Eppendorf tubes containing 100 pmoles of...
3H-folic acid (specific radioactivity 2,000 disintegrations per minute/pmole) in the absence or presence of increasing concentrations of natural folate or folate antagonists. Following 10 minutes at 4°C, cells were centrifuged in an Eppendorf centrifuge (30 seconds at 10,000 revolutions per minute), the supernatant was aspirated, and cell pellets were resuspended in 200 μl of water and analyzed for radioactivity (Optima Gold scintillation fluid; United Technologies/Packard, Brussels, Belgium). Nonspecific binding of 3H (usually <2% of specific binding) was determined by measuring cell-associated radioactivity in the presence of a 1,000-fold molar excess of unlabeled folic acid. Concentrations of natural folates and selected folate antagonists required to displace 50% of 3H-folic acid from FR were determined and are presented as binding affinities relative to folic acid. For comparison, data for affinities of the RFC for natural folates and folate antagonists from previous studies (16,27) are included for comparison.

Cell proliferation inhibition assay. CHO/WT cells and CHO/FR<sub>β</sub> cells were seeded in individual wells of a 24-well tissue culture plate at a density of 1 × 10<sup>4</sup>/cm<sup>2</sup>. After 24 hours, 8 concentrations (2.5-fold increments) of folate antagonists were added in the absence or presence of 1 μM folic acid (to block FR) or 20 nM leucovorin. After 72 hours of incubation, cells were harvested by trypsinization and counted for cell viability as described elsewhere (40).

In other experiments, human monocytic macrophage THP-1 cells were tested for antiproliferative effects of folate antagonists. After 72 hours, drug-exposure cell counts were performed with a hemocytometer, and viability was determined by trypan blue exclusion.

RESULTS

FR<sub>β</sub> synovial tissue immunohistochemistry. Immunohistochemical staining of all RA synovial tissues showed high levels of FR<sub>β</sub> expression in the intimal lining layer as well as the synovial sublining. The staining pattern for FR<sub>β</sub> was consistent with 3A5 (macrophage) staining, whereas T cell areas showed no staining (Figures 1A–C). In fact, more-detailed fluorescence microscopic analysis demonstrated colocalization of FR<sub>β</sub> and CD68 (macrophage marker) on the cellular membranes of synovial tissue–infiltrating macrophages and intimal macrophages (Figures 1D–F). In noninflammatory synovial tissue from orthopedic controls, no staining for FR<sub>β</sub> was observed, consistent with low numbers of macrophages (results not shown).

Staining results were examined by computer-assisted digital image analysis. A significant correlation (P = 0.004) between 3A5 and FR<sub>β</sub> expression in the synovial sublining layer (number of cells/mm<sup>2</sup>) was found (Figure 2A). The median number of positive cells in the synovial sublining layer was 126/mm<sup>2</sup> (range...
9–630) for FRβ and 219/mm² (range 11–622) for 3A5. The median number of macrophages decreased after 4 months of treatment with MTX (from 219 cells/mm² to 119 cells/mm² in the synovial sublining layer; \( P < 0.14 \)). FRβ expression after 4 months of treatment with MTX correlated positively (\( r = 0.32 \)) with improvement in the DAS28, but this did not reach statistical significance (\( P = 0.12 \)) (Figure 2B).

**FRβ mRNA expression in RA synovial tissue and peripheral blood cells of RA patients.** To further confirm the differential expression of FRβ in synovial tissue, levels of mRNA for FRβ were determined by PCR analysis in synovial tissue biopsy samples as compared with those in peripheral blood cells from RA patients, including lymphocytes, ex vivo–activated T cells, peripheral blood monocytes, and ex vivo monocyte–derived macrophages. FRβ mRNA levels are presented relative to expression in CHO/FRβ cells (30), which was set at 100%. The ranking of FRβ mRNA expression was highest in RA synovial tissue (median 17% as compared with CHO/FRβ cells), followed in descending order by expression in ex vivo monocyte–derived macrophages (3.4%), PBLs (0.7%), monocytes (0.03%), and ex vivo–activated T cells (0.004%) (Figure 3).

**Binding affinities of FRβ versus FRα for folate antagonists.** The binding affinities of FRα for selected folate antagonists have previously been reported (16), but the extent to which they overlap or differ from the binding affinities of the FRβ isoform has not been established. To this end, the binding affinities (relative to folic acid) of FRβ versus FRα to a series of folate-based inhibitors of DHFR, TS, and GART were determined. The results are shown in Figure 4.

Compared with folic acid, both FRβ and FRα displayed a rather low affinity for the group of DHFR inhibitors. Binding affinity of FRβ for MTX was ~50-

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![Figure 2](image_url)

**Figure 2.** A, Correlation between the numbers of 3A5-positive (macrophages) and folate receptor β (FRβ)–positive cells per mm² in synovial tissue from rheumatoid arthritis (RA) patients (n = 15) before treatment with methotrexate (MTX), by linear regression analysis. The median number of positive cells in the synovial sublining layer was 126/mm² (range 9–630) for FRβ and 219/mm² (range 11–622) for 3A5. B, Correlation between improvement in the Disease Activity Score 28-joint assessment (ΔDAS28) and in FRβ expression on macrophages (number of positive cells/mm²) from RA patients after 4 months of treatment with MTX, by linear regression analysis.

![Figure 3](image_url)

**Figure 3.** Folate receptor β (FRβ) mRNA expression in synovial tissue and ex vivo–cultured rheumatoid arthritis (RA) peripheral blood lymphocytes (PBLs). Median FRβ mRNA expression was determined in ex vivo–activated (Act.) T cells (n = 22), monocytes (n = 9), PBLs (n = 9), ex vivo monocyte–derived macrophages (n = 25), and synovial tissue samples (n = 7) from RA patients. FRβ expression in these samples is shown as a percentage of the expression in FRβ-transfected Chinese hamster ovary cells (CHO/FRβ), which was set at 100% (horizontal line). Values are the median and range.
Folate antagonist–induced growth inhibition of FRβ-expressing cells. To investigate whether the folate antagonists tested in the current study would convey potential growth-inhibitory effects against macrophage-like cells, this parameter was investigated in human monocytic macrophage THP-1 cells (Table 1). Consistent with the RFC as the dominant transport route in THP-1 cells, potent growth-inhibitory effects were observed for all folate antagonists, except CB300635 and BCG 945, both of which have a poor affinity for the RFC (25,27).

Three folate antagonists for which FRβ displayed the highest binding affinity (CB300635, AG2034, and BCG 945) were evaluated for their potency to target FRβ by provoking cell growth inhibition of CHO/FRβ cells. Against CHO/WT cells, BCG 945 induced growth inhibition only at extracellular concentrations >1,000 nM (Figure 5A). Remarkably, growth inhibition of CHO/FRβ cells was induced at markedly lower concentrations (10–50 nM) of BGC 945. The addition of folic acid to these cell cultures completely abrogated the activity of BGC 945, consistent with a blockade of FRs. Despite displaying the highest binding affinity for FRβ, CB300635 was not markedly potent in inducing growth inhibition in CHO/FRβ cells (Figure 5B). AG2034 may use both constitutively expressed RFC and FRs as the route for cell entry (Figure 4). Thus, AG2034 displayed growth-inhibitory potential against CHO/WT cells and

Table 1. Growth inhibitory effects of folate antagonists on human monocytic macrophage THP-1 cells*

<table>
<thead>
<tr>
<th>Folate antagonist</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR inhibitors</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>PT523</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>PT644</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>TS inhibitors</td>
<td></td>
</tr>
<tr>
<td>GW1843</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Pemetrexed</td>
<td>10.7 ± 2.2</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>BGC 9331</td>
<td>8.7 ± 0.8</td>
</tr>
<tr>
<td>CB300635</td>
<td>4,850 ± 285</td>
</tr>
<tr>
<td>BGC 945</td>
<td>3,630 ± 350</td>
</tr>
<tr>
<td>GART inhibitors</td>
<td></td>
</tr>
<tr>
<td>DDATHF</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>AG2034</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

* THP-1 cells were grown in RPMI 1640 medium containing 2.2 µM folic acid to block folate receptor (FR) activity and then exposed to folate antagonists for 72 hours. Values are the mean ± SD concentration of folate antagonist required to displace 50% of ^3H-folic acid from FRβ or FRα (50% inhibition concentration [IC_{50}]), as determined in 3 separate experiments. DHFR = dihydrofolate reductase; TS = thymidylate synthase; GART = glycinamid ribonucleotide transformylase; DDATHF = 5,10-dideazatetrahydrofolate.
to a greater extent against CHO/FRbeta cells. Consistent with these findings, abrogation of the growth-inhibitory effects of AG2034 by FRbeta blocking (with folic acid) and by RFC blocking (with leucovorin) were only partial (Figures 5C and D).

DISCUSSION

Since MTX is the anchor drug in many therapeutic regimens for RA, delineation of the genetic, biochemical, and metabolic parameters that could assist in predicting and/or improving the therapeutic response to MTX have received considerable interest recently (1,19,41,42). In this study, we focused specifically on the role of cell membrane transport of MTX, which, in activated synovial macrophages, is mediated predominantly by the folate receptor beta isoform (11). Given the notion that the molecular and functional properties of FRs and the constitutively expressed RFC differ considerably, a better understanding of the properties of FRbeta may facilitate a better therapeutic window by selective targeting of FRbeta over the RFC.

We showed that FRbeta expression primarily colocalized with macrophages in the intimal lining layer and the sublining of synovial tissues from RA patients and may therefore be an attractive target for folate antagonists. It has previously been reported that both the FRalpha and FRbeta isoforms exhibit a rather low affinity for MTX as compared with folic acid (16,43). In this context, it would be worthwhile to investigate whether folate antagonists with a higher binding affinity to FRs would provide a greater degree of selectivity and thereby elicit a potentially improved therapeutic response. Screening for the binding affinities of a series of second-generation folate antagonists, some of which have proven anticancer activity (18), revealed that the DHFR inhibitors had a rather low FRbeta affinity. This is consistent with the previously reported structure/activity relationship, demonstrating that the alpha isoform of FR had low affinities for folate antagonists that had a 2,4-diamino–based structure. (Illustrations of the chemical structures of these folate antagonists are available online at http://jwvanderheijden.blogspot.com/.)

Interestingly, while FRalpha demonstrated a relatively high binding affinity for all of the folate-based inhibitors of thymidylate synthase we tested, for FRbeta, this was retained only for 3 compounds that share a common chemical property of 3-ring structures and/or glutamate side-chain modifications (CB300635, GW1843, and BCG 945). The latter modification also markedly suppressed its ability to be transported via the RFC and, thus, contributed to greater FR selectivity. In fact, selective targeting of BCG 945 for FRalpha and not the RFC has been demonstrated in FRalpha-overexpressing tumor cell lines. (17,27). In addition to folate-based TS
inhibitors, FRβ also exhibited moderate to high binding affinities for the folate-based GART inhibitors AG2034 and DDATHF, which classifies them as folate antagonist drugs that can be transported both via the RFC (16) and via FRs.

FRβ-transfected CHO cells were used as a model system in which to evaluate the efficiency of FRβ-mediated cellular uptake of folate antagonists by conveying antiproliferative effects (44). This cell line model may be clinically representative, based on [3H]-folic acid binding levels and FRβ mRNA levels that are consistent with the FRβ mRNA levels in RA synovial tissue (Figure 3).

The largest differential in activity between control (RFC-expressing) CHO cells and FRβ-transfected cells was observed for BCG 945, consistent with a poor affinity for transport via the RFC and a high affinity for binding FRβ. The FRβ-mediated uptake of BCG 945 could be inhibited by blocking the receptor with excess folic acid, implying that circulating natural folates in synovial tissue/plasma could attenuate the potential activity of BCG 945 in vivo either by receptor occupancy/competition or by receptor down-regulation. In this context, it may be anticipated that, for example, fortification of food with folate may reduce the activity of this folate antagonist, whereas restrictions in dietary folate intake could further enhance the therapeutic efficacy of these types of drugs (45).

An intriguing observation was that the compound CB300635, for which FRβ had the highest affinity (1.5-fold higher than folic acid), was not differentially active against FRβ-transfected cells. This may suggest that the high-affinity binding may have a downside, which is the poor release of the compound from the receptor in an acidic compartment during endocytic cycling of the receptor (46). Hence, to allow optimal targeting of FRβ by folate antagonists, both conditions—efficient binding to the receptor and efficient intracellular release from the receptor for target delivery—should be fulfilled.

The GART inhibitors DDATHF and AG2034 showed dual specificity for the RFC and FRα/β as folate transport systems. Whether this property renders a different type of activity profile as compared with FR-selective compounds awaits further investigation. In this respect, 2 recent studies (47,48) showed that 2 folate-based GART inhibitors could confer suppression of adjuvant-induced arthritis in rats and collagen-induced arthritis in mice. Moreover, in a preliminary study, we showed that novel generation of folate antagonists were potent suppressors of TNFα production by activated T cells from RA patients (49).

Although MTX is the drug of first choice in the treatment of RA, there is room for improving the efficacy of MTX, for example, in clinical cases of primary and acquired failure of MTX therapy (19). Further evaluation of folate antagonists with properties of high binding affinity for FRβ and low affinity for the RFC, which are prototypically harbored by BCG 945 (27), may pave the road for a more selective targeted therapy of activated synovial macrophages. Even in cases where FRβ expression would be low, there may be strategies for increasing FRβ expression by pretreatment with retinoids and/or histone deacetylase inhibitors (39,50,51). Thus, beyond exploiting FRβ for delivery of therapeutic agents or for imaging purposes (12,14), the present study supports therapeutic strategies that include FRβ-mediated uptake of clinically active second-generation folate antagonists.

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AUTHOR CONTRIBUTIONS

Drs. Van der Heijden and Jansen had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study design. Van der Heijden, Oerlemans, Dijkmans, Jackman, Kraan, Ratnam, Jansen. Acquisition of data. Van der Heijden, Oerlemans, Qi, van der Laken, Jackman, Kraan, Tak, Ratnam, Jansen. Analysis and interpretation of data. Van der Heijden, Oerlemans, Dijkmans, Qi, Lems, Jackman, Tak, Ratnam, Jansen. Manuscript preparation. Van der Heijden, Oerlemans, Dijkmans, Qi, van der Laken, Lems, Jackman, Kraan, Tak, Ratnam, Jansen. Statistical analysis. Van der Heijden, Oerlemans, Jansen.

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