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Metabolism-blocked antifolates as potential anti-rheumatoid arthritis agents: 4-Amino-4-deoxy-5,8,10-trideazapteroyl-D,L-4′-methyleneglutamic acid (CH-1504) and its analogs

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ABSTRACT

4-Amino-4-deoxy-5,8,10-trideazapteroyl-D,L-4′-methyleneglutamic acid (CH-1504) is the prototype of a potentially therapeutically more selective class of antifolates for rheumatoid arthritis treatment. This class is characterized by retention of dihydrofolate reductase (DHFR; EC 1.5.1.3) as their locus of action and transport by the reduced folate carrier (RFC; SLC19A1), but their lack of metabolism by known pathways of antifolate (e.g., methotrexate (MTX)) metabolism. Five new CH-1504 analogs (CHL-001–CHL-005) were synthesized and diastereomers of CH-1504 itself were obtained by preparative chiral HPLC; all were characterized biochemically. The analogs are not metabolized by aldehyde oxidase (EC 1.2.3.1), carboxypeptidase G2 (EC 3.4.17.11), or (excepting CHL-003) folylpolyglutamate synthetase (EC 6.3.2.17) and thus, unlike MTX, are ''metabolism-blocked''. All analogs are potent DHFR inhibitors; several are nearly as potent as MTX or CH-1504. Each analog uses the RFC for transport, although with varying apparent affinities. In contrast, each weakly inhibits other enzymes of folate metabolism relevant to rheumatoid arthritis therapy (thymidylate synthase (EC 2.1.1.45), two formyltransferases of purine biosynthesis (EC 2.1.2.2 and EC 2.1.2.3), and 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20)). Biochemical characterization showed one 4′-diastereomer of racemic CH-1504 was significantly more active than the other. Based on literature data concerning the effect of D- and L-glutamic acid substitution on antifolate activity, it is likely that the diastereomer containing L-4′-methylene-glutamic acid is the more active. Because of concern about potential pharmacokinetic and biochemical effects of D-4′-methylene-glutamic acid-containing species, these data suggest that future analogs should contain only L-4′-methylene-glutamic acid. Overall, these data provide several interesting new leads for preclinical development.

1. Introduction

Rheumatoid arthritis (RA) is a potentially debilitating autoimmune disease currently afflicting about 2.1 million people in the U.S. (http://www.arthritis.org). RA can lead to progressive disability, decreased life expectancy, and psychological trauma; treatment toxicity and costs place a further burden on afflicted individuals. Characteristics of RA include painful chronic synovitis in affected joints and the presence in synovial fluid of immune effector cells. In addition to localized

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effects, RA also causes systemic symptoms such as fatigue and malaise [1]. The pain and stiffness of RA may be alleviated by nonsteroidal anti-inflammatory drugs (NSAIDs, e.g., aspirin), but these agents do not treat the underlying condition and bone and cartilage erosion may occur. After diagnosis, patients generally begin treatment with disease modifying anti-rheumatic drugs (DMARDs). The antifolate methotrexate (MTX; 4-NH_2-10-CH_3-folic acid; 4-NH_2-10-CH_3-PteGlu) is currently considered an agent of choice when a DMARD is indicated based on efficacy and cost considerations [2,3]. MTX is highly effective in treating RA in patients who respond. However, less than 70% of patients respond [1] and a large percentage of patients become intolerant as a result of the side effects (principally GI distress, hematological toxicity and hepatotoxicity) of this drug. Other options for treating RA, such as biologicals (e.g., Etanercept), are now available but are costly, may require inconvenient injection delivery, and each has its own set of side effects [4], some of which (such as increased infection) are serious. Because MTX is effective in RA therapy, discovery of an antifolate that retains the anti-RA efficacy of MTX but has diminished side effects would represent an important advance in RA treatment.

The therapeutic anti-inflammatory mechanism of MTX in RA has not yet been convincingly elucidated, although evidence for multiple proposed mechanisms has been presented. These mechanisms include: (a) immune cells responsible for synovial inflammation are killed; (b) local release of pro-inflammatory cytokines is decreased; (c) local release of anti-inflammatory cytokines is increased; and/or (d) metalloproteinase activity causing cartilage degradation is inhibited [1]. Likewise the mechanisms of MTX toxicity are not clearly delineated. However, chronic toxicity is likely caused by long-term MTX accumulation in tissues of therapy limiting toxicity (e.g., liver [5–7] and bone marrow [8]) and may be related to depletion of reduced folates [9,10]. MTX poly(γ-glutamyl) metabolites are the primary contributors to long-term MTX retention [6,11]. The role of MTX polyglutamates in therapeutic efficacy in RA is controversial with some studies showing they are required (e.g. [12]), while other studies (e.g. [13]) show they are not essential and may even contribute to toxicity, including hepatotoxicity [14] and bone marrow suppression [15]. These latter studies provide a rationale for suggesting that an MTX analog that cannot form polyglutamates, such as CH-1504 [16], may be therapeutically superior to MTX in treating RA.

Other metabolites of MTX may also interfere with either its pharmacodynamic action or make its pharmacokinetics less predictable. For example, MTX is known [17] to undergo hydroxylation by aldehyde oxidase (EC 1.2.3.1) to form 7-hydroxy-MTX (7-OH-MTX), even at the low doses used in RA treatment [18]. This metabolite is less soluble than MTX [19], is >100-fold less potent as an inhibitor of dihydrofolate reductase (DHFR) [20], the enzyme target of MTX, and is ≈100-fold less potent as a growth inhibitor [17]. Pharmacogenetic variations in aldehyde oxidase expression [21] or differences in the ability to excrete 7-OH-MTX may lead to unpredictable toxicity. Likewise some MTX is secreted into bile from the liver and reabsorbed in the intestine [22]. This enterohepatic circulation may be interrupted by metabolism in the intestinal lumen by gut bacteria secreting a carboxypeptidase G (EC 3.4.17.11)-like activity [23] that removes the glutamate intrinsic to the MTX structure [24]. Similar to 7-hydroxylation, deglutamylation decreases solubility, reduces re-uptake, and decreases inhibitory potency [24]. Differences in the pharmacogenetic status of the patient with respect to aldehyde oxidase and variations in intestinal flora (from diet or antibiotic use) may contribute to differences in pharmacokinetics of MTX and could contribute to toxicity and/or a decrease in efficacy in RA therapy. Accordingly, antifolates that do not undergo either type of metabolism may be therapeutically superior to MTX. Since side effects cause the discontinuance of treatment by a significant fraction of RA patients who start MTX therapy [1,25], a replacement with equal efficacy but lower side effects would be a significant advance.

CH-1504 (Fig. 1; formerly known as AA-243 or Mobiletrex [16]), a potent antifolate that inhibits dihydrofolate reductase (DHFR; EC 1.5.1.3), was designed to be "metabolism-blocked" for polyglutamylation, 7-hydroxylation, and deglutamylation. This antifolate is a more potent growth inhibitor than is MTX itself [16]. Because of its unique properties, this analog was chosen for development as a new anti-RA agent. In a non-controlled, non-blinded study of 20 RA patients [26,27], CH-1504 was superior to MTX both in terms of efficacy and tolerability. Although this study was not optimized, it provided strong support for further study of this class of antifolates in RA. Phase 1 studies on single and multiple oral doses of CH-1504 have been performed [28] and showed that CH-1504 is well tolerated at doses expected to be therapeutically effective. Phase II efficacy studies began early in 2008. Other antifolates that are blocked only for polyglutamylation have also shown therapeutic selectivity in preclinical RA models [29].

CH-1504 is a proof-of concept agent that metabolism-blocked antifolates can have greater efficacy than MTX in RA. As such, it serves as a platform from which to design agents that have even higher therapeutic efficacy. As a first step in that effort, a number of CH-1504 analogs (Fig. 2) have been designed, synthesized, and characterized. In addition, the 4′-diastereomers of CH-1504, which result from use of racemic DL-4′-methylene-glutamic acid (4′-methylene-Glu) (Fig. 1) in its synthesis, have been resolved and characterized. The results show that some of these CH-1504 analogs have properties that encourage further development in preclinical models of RA. The diastereomers of CH-1504 have divergent biochemical

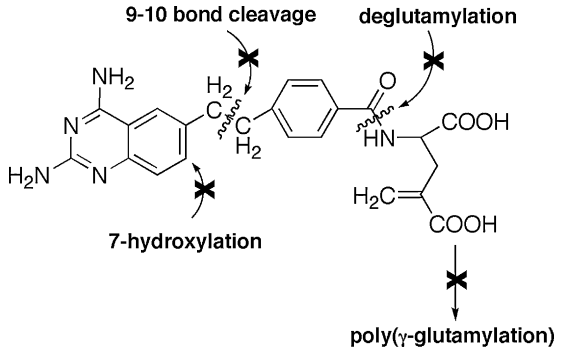

Fig. 1 – Structure of metabolism-blocked antifolates, as exemplified by CH-1504.

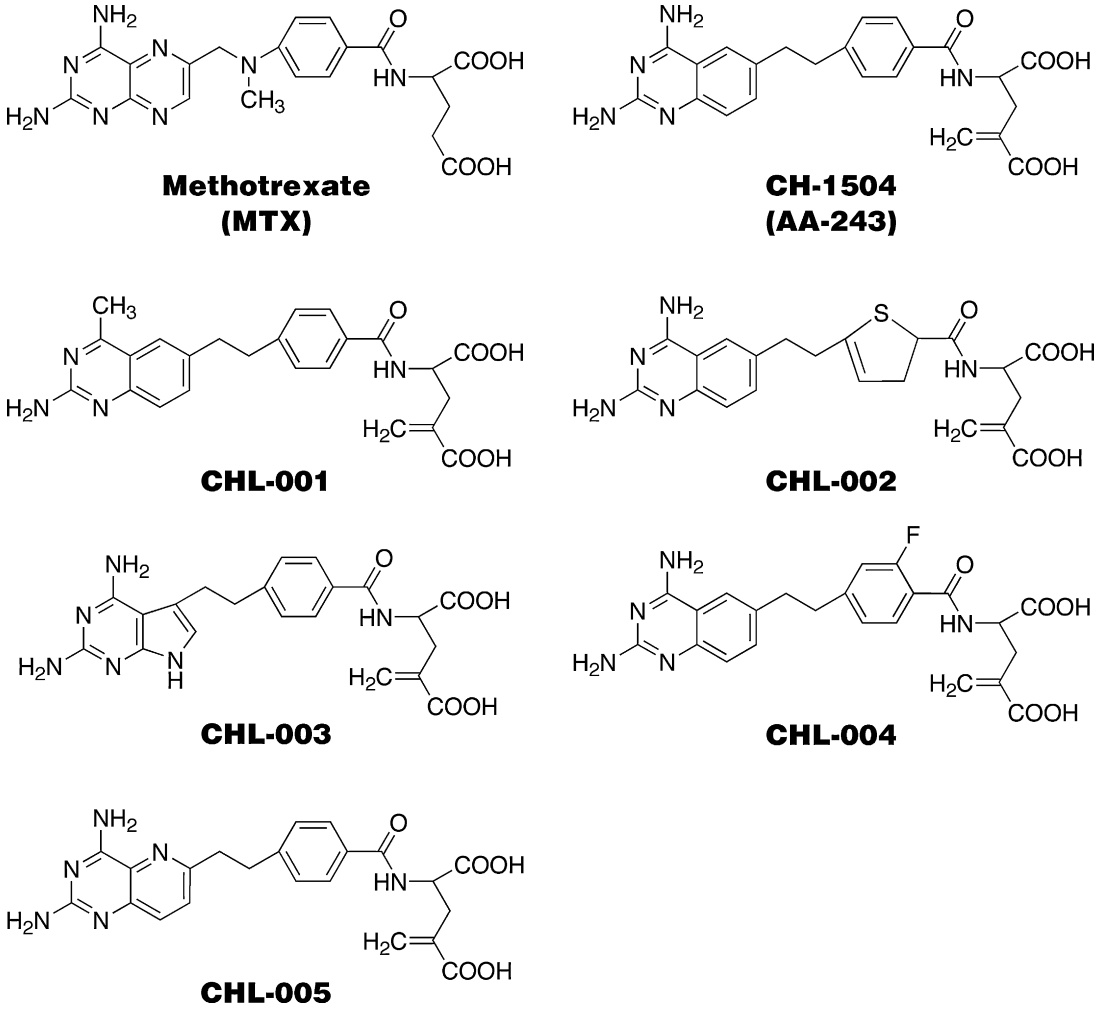

Fig. 2 – Structures of methotrexate (MTX), CH-1504 (a.k.a. AA-243), CHL-001, CHL-002, CHL-003, CHL-004, and CHL-005.

properties suggesting that the single, more active L-4′-methylene-Glu-containing diastereomer could potentially have therapeutic properties superior to the racemic mixture that is currently in Phase II clinical testing.

2. Materials and methods

2.1. Materials

HPLC grade acetonitrile was from Fisher Scientific (Fair Lawn, NJ). 2-Mercaptoethanol was from Acros Organics (Geel, Belgium). All common chemicals were of reagent grade or higher. [^{3}H]MTX (12–52 Ci/mmol; Moravek Biochemicals, Brea, CA) was used without purification as long as HPLC analysis (below) showed >95% purity, because at this purity level intracellular ^{3}H was >90% associated with MTX or its metabolites (below). Purity analysis was identical to that used for intracellular radiolabel and was capable of resolving likely ^{3}H-containing synthetic/chemical decomposition/radiolysis side-products including H_2O, p-aminobenzoate, p-aminobenzoylglutamate, N-CH_3-p-aminobenzoylglutamate, 10-CH_3-folic acid, and aminopterin from MTX.

2.2. Antifolates

Methotrexate (MTX; Fig. 1) was obtained from Tocris Bioscience (Ellisville, MO). Aminopterin (AMT; 10-desmethyl-MTX; 4-NH_2-PteGlu) was obtained from Sigma Chemical (St. Louis, MO). A sample of AA-243 (Mobiletrex) was obtained from Dr. M.G. Nair. Clinical grade CH-1504 (Fig. 1) containing DL-4′-methylene-Glu was synthesized as described [16] and provided by Chelsea Therapeutics International, Ltd. (Charlotte, NC). Novel CH-1504-related antifolates (Fig. 2) were provided by Chelsea Therapeutics International, Ltd. (Charlotte, NC) and were synthesized by Syntagon AB (Södertälje, Sweden; synthetic details are provided in Supplementary Materials). LC-mass spectrometry, ^{1}H NMR, and ^{13}C NMR spectra were consistent with the structures shown. All analogs were >95% pure by vendor HPLC analysis. Molecular weights (MW) of analogs were derived using the "Calculate MW" function of ChemDraw 7.0.3 (CambridgeSoft, Cambridge, MA), assuming compounds were free acids, unhydrated, and contained no solvent.Extinction coefficients were determined by weighing an exact amount of drug (11–13 mg) on a microbalance, quantitatively transferring and suspending it in 8 ml ultrapure water, and by solubilizing the compound by adjusting the pH to a value near neutral by

addition of known amounts of NaOH solutions in ≤100 μL aliquots to avoid highly alkaline conditions. Based on the mass, calculated MW (above), final volume and the 220–370 nm absorption spectrum, extinction coefficients were derived at pH 1, 7, and 13 as follows: *CH-1504* (pH 1, λ_{max} 236 nm (41,400); pH 7, λ_{max} 236 nm (41,800); pH 13, $\lambda_{max\text{-}1}$ 235 nm (44,600), $\lambda_{max\text{-}2}$ 340 nm (9,200)); *CHL-001* (pH 1, $\lambda_{max\text{-}1}$ 232 nm (37,900), $\lambda_{max\text{-}2}$ 249 nm (40,200); pH 7, λ_{max} 241 nm (49,900); pH 13, λ_{max} 241 nm (50,000)); *CHL-002* (pH 1, λ_{max} 233 nm (42,300); pH 7, λ_{max} 233 nm (43,500); pH 13, $\lambda_{max\text{-}1}$ 232 nm (49,000), $\lambda_{max\text{-}2}$ 278 nm (21,400)); *CHL-003* (pH 1, λ_{max} 234 nm (27,500); pH 7, λ_{max} 229 nm (27,100); pH 13, $\lambda_{max\text{-}1}$ 228 nm (28,200)); *CHL-004* (pH 1, λ_{max} 236 nm (39,000); pH 7, λ_{max} 235 nm (39,700); pH 13, $\lambda_{max\text{-}1}$ 233 nm (42,700)); and *CHL-005* (pH 1, $\lambda_{max\text{-}1}$ 223 nm (29,600), $\lambda_{max\text{-}2}$ 321 nm (5900); pH 7, λ_{max} 235 nm (27,100); pH 13, $\lambda_{max\text{-}1}$ 238 nm (28,800), $\lambda_{max\text{-}2}$ 338 nm (3900). Extinction coefficients for MTX and aminopterin were from the literature [30].

2.3. Preparation of CH-1504 4′-diastereomers

CH-1504 4′-diastereomers were resolved preparatively (Peaks 1 and 2) by Advanced Separations Technologies Inc. (Whippany, NJ) on a Chirobiotic™ T 10 μm column (3 × 25 cm) in 0.25% (w/w) methanol in ammonium acetate, essentially as described by Nair and Kisliuk [31]. Analytical chiral chromatography (4.6 mm × 100 mm Chirobiotic™ T 5 μm column; same solvent at 1.5 ml/min) showed that racemic CH-1504 contained 2 major peaks of essentially equal area (50.2:49.8). Similar analyses showed that resolved Peak 1 contained 2.7% of Peak 2, while resolved Peak 2 contained 3.2% of Peak 1. Fractions containing Peak 1 or Peak 2 were pooled individually and concentrated by rotary evaporation at 50 °C under vacuum. Each concentrate was acidified with HCl to pH 4 [16] and addition of ultra-pure water enhanced precipitation of the pale yellow solids. Individual products were isolated by filtration and dried under vacuum at 40 °C. Both Peak 1 and Peak 2 were obtained as cream/light tan powders. When the final products were solubilized and adjusted to pH 8.0 (standard for CH-1504), solutions of both Peaks 1 and 2 were turbid. Attempted removal of the turbidity by centrifugation yielded a cloudy top layer (lower density) rather than a pellet. The turbidity was retained by a Centricon™ C30 centrifugal concentrator (Amicon, Beverly, MA) centrifuged at 3450 × g_{max} for 15 min at room temperature. The resulting pass-through fraction of both Peaks 1 and 2 were clear and their UV–visible spectra at pH 1, 7, and 13 were identical to racemic CH-1504. The absence of this insoluble material in the racemic CH-1504 used for purification, the low density of the insoluble material and its removal by a membrane filter with a 30 kDa nominal cut-off is consistent with its being derived from the resolution process. The column used to resolve the diastereomers utilizes teicoplanin as the chiral selector. Since teicoplanin has a methyldecanoic acid lipid side-chain, hydrolysis of the lipid amide linkage would release lipid. The buoyancy of the insoluble material is consistent with this possibility.

2.4. Enzymes and enzyme activity assays

Dihydrofolate reductase (DHFR; EC 1.5.1.3), thymidylate synthase (TMPS; EC 2.1.1.45), 5-amino-4-imidazolecarboxamide ribotide (AICAR) formyltransferase (AICARFT; EC 2.1.2.3), and glycinamide ribotide (GAR) formyltransferase (GARFT; EC 2.1.2.2) were partially purified from CCRF-CEM human T-lymphoblastic leukemia cells as described [32]. Recombinant human cytosolic folylpolyglutamate synthetase (FPGS; EC 6.3.2.17) was expressed in Sf9 insect cells and purified as described [33]. Aldehyde oxidase (AO; EC 1.2.3.1) was purified from rabbit liver [17]. Pure carboxypeptidase G2 (CPG2; EC 3.4.17.11) from *Pseudomonas* sp. strain RS-216 was a gift of the late Dr. Roger Sherwood (Microbial Technology Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, England) [34]. Recombinant human 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) reductase (MTHFR; EC 1.5.1.20) was a generous gift of Drs. Rowena Mathews and C. Lee Elmore, University of Michigan, Life Sciences Institute, Ann Arbor, MI.

All enzymes were assayed using a Beckman DU640 at 37°, except as noted. Activity was linear with respect to time over the assay period and the level of enzyme used was verified to be in the linear range, except as noted. Inhibitory potency was assayed by adding increasing concentrations of an antifolate to standard assays and measuring residual activity. If inhibition observed at 50 μM was >50%, IC_{50} (concentration of analog inhibiting enzyme or transport activity by 50% relative to untreated control) values were determined; otherwise the IC_{50} was concluded to be >50 μM. IC_{50} values were interpolated from plots of activity relative to untreated control versus inhibitor concentration by performing a linear regression of the two data points on either side of 50% relative activity and calculating the inhibitor concentration corresponding to 50% relative activity.

DHFR activity was assayed over 4 min at 20 μM dihydrofolate (DHF) and 50 μM NADPH as described [32]. Apparent activity in the presence of either cosubstrate alone (DHF or NADPH) was ≤3% of activity in the presence of both substrates. Inhibitory potency was assessed by pre-incubating DHFR (1.9×10^{-3} I.U.) in the presence of NADPH with five graded concentrations of inhibitor for 3 min at 37°, initiating the reaction by addition of DHF, and quantitating residual DHFR activity.

AO was quantitated [17] using 100 μM MTX as substrate using a molar extinction coefficient of 9000 M^{-1} cm^{-1} at 341 nm [35]. EDTA is normally present in AO assays, however EDTA was found to contribute a large early eluting peak in subsequent HPLC analysis (below) of AO reaction mixtures. For that reason, EDTA was not present in the assays used to examine substrate activity of analogs (or MTX) because the same reaction mixtures were utilized for HPLC analysis. Elimination of EDTA from standard AO assays led to only an 8% decrease in activity. Since it was unknown what spectral change would occur if a CH-1504 analog were an AO substrate, substrate activity of the analogs was examined by two alternative methods. For the spectral assay, an amount of MTX or analog that gave 2–2.5 absorbance units at the highest λ_{max} was added to a standard AO reaction mixture; final concentrations were MTX (80 μM), CH-1504 (50 μM), CHL-001 (60 μM), CHL-002 (50 μM), CHL-003 (75 μM), CHL-004 (50 μM), and CHL-005 (60 μM). AO-catalyzed spectral changes (220–370 nm) of MTX and analogs were followed by repetitive scanning at appropriate time intervals after initiation with AO (20–28 I.U.). It is likely that hydroxylation of CH-1504 analogs

would produce a change in 220–370 nm spectral properties, as observed with MTX, AMT, and other 2,4-diamino antifolates. CH-1504 analogs were examined at 2-fold longer reaction times than MTX to increase assay sensitivity. As noted in the text, although MTX was allowed to react for ≈45 min, its reaction half-time was ≈2 min. The second method for detecting hydroxylation relied on HPLC analysis according to a minor modification (to maintain significant parent analog retention) of our standard HPLC method (below). Samples ±AO used in the spectral assay (above) were frozen at −80° after assay. Just before HPLC analysis, samples were thawed and filtered (Millipore Amicon MP-3; YMT, 30 kDa cut-off).CPG2 activity was assayed and quantitated by following the absorbance change at 320 nm as MTX is deglutamylated to 4-amino-10-methyl-pteroic acid (DAMPA; desglutamyl-MTX) (8300 M^{-1} cm^{-1}) [36]. Spectral changes during CPG2 treatment of MTX and analogs were followed by repetitive scanning (220–370 nm) at appropriate time intervals. CH-1504 analogs were incubated at 10-fold higher CPG2 levels (≈0.07 I.U.) and for twice the time (60 min versus 30 min) as MTX (≈0.007 I.U.) to increase sensitivity. For the spectral assay, an amount of analog that gave ≈2 absorbance units at its λ_{max} was used; final concentrations were MTX (60 μM), CH-1504 (60 μM), CHL-001 (60 μM), CHL-002 (60 μM), CHL-003 (75 μM), CHL-004 (50 μM), and CHL-005 (60 μM). After reaction, samples were rapidly frozen and stored at −80 °C until HPLC analysis, similar to that for AO (above).

FPGS was assayed as described [33]. AMT (100%; assay standard), CH-1504 (99–101%), CHL-001 (95–97%), CHL-002 (102–103%), CHL-003 (98–100%), CHL-004 (98–99%), CHL-005 (98–100%) were each recovered nearly quantitatively during duplicate mock assays, thus ensuring that polyglutamate products would also be quantitatively recovered. A complete kinetic analysis for AMT (4–40 μM range) was performed in each experiment; linearity of FPGS activity with respect to time was verified at the lowest and highest AMT concentrations. FPGS substrate activity of each CH-1504 analog was tested at 10 and 100 μM only, unless substrate activity was observed (this only occurred for CHL-003). Inhibition of FPGS by CH-1504 analogs was assayed by including 100 μM analog simultaneously with 40 μM AMT. The percent inhibition was calculated relative to a control containing only AMT.

TMPS was assayed over 30–40 min by measuring ^3H displacement from [5-^{3}H]dUMP into $^3H_20$ essentially as described [37,38], except that pure (6*R*,*S*)-5,10-CH_2-H_4PteGlu (Schircks, Jona, Switzerland) was used. Apparent activity in the absence of (6*R*,*S*)-5,10-CH_2-H_4PteGlu was ≤0.6% of activity in the presence of both substrates. Inhibitory potency of analogs was assessed by introducing up to five graded concentrations of analog into reaction mixtures, initiating the reaction by addition of TMPS ($2.8 \pm 0.1 \times 10^{-5}$ I.U.), and quantitating residual TMPS activity.

The AICARFT assay [39] follows the increase in 298 nm absorbance over 4 min as 10-formyl-tetrahydrofolic acid (10-f-H_4PteGlu) is converted to 5,6,7,8-tetrahydrofolic acid (H_4PteGlu) when AICAR is formylated to formyl-AICAR. Apparent activity in the presence of either 10-f-H_4PteGlu or AICAR alone was ≤2% of activity in the presence of both substrates. Inhibitory potency of analogs was assessed by pre-incubating AICARFT (1.6×10^{-3} I.U.) in the presence of 10-f-H_4PteGlu with analog for 3 min, initiating the reaction by addition of AICAR, and quantitating residual AICARFT activity.

GARFT was assayed over 3 min by adapting the AICARFT assay (above; [39]) by replacing AICAR with glycinamide ribonucleotide (GAR), changing the reaction temperature to 25 °C, and using the reactions conditions of Caperelli [40]. GAR was a generous gift of Dr. Carol Caperelli (College of Pharmacy, University of Cincinnati Medical Center, Cincinnati, OH). Apparent activity in the presence of either 10-f-H_4PteGlu or GAR alone was ≤1% of activity in the presence of both substrates. Inhibitory potency of analogs against GARFT (1.0×10^{-3} I.U.) was quantitated as described for AICARFT.

MTHFR was assayed at 25° as described by Yamada et al. [41], except that 100 μM (6*R*,*S*)-5,10-methylenetetrahydrofolate (Schircks, Jona, Switzerland; solubilized as described by the manufacturer) was used as the folate substrate.

2.5. [^{3}H]MTX transport inhibition

Transport of 2 μM [^{3}H]MTX at 37° by intact CCRF-CEM human T-cell leukemia cells was assayed by a micro-method [42] utilizing repeated iced saline washes to remove extracellular drug. Intracellular radiolabel in control cells was analyzed by HPLC (below) and was typically >90% MTX and its metabolites (in one duplicate of one experiment it was 79%). Because transport inhibition is measured after only 5 min of [^{3}H]MTX uptake, radioactivity levels in control cells are low. Thus <750 cpm were injected on the HPLC leading to stochastic low apparent "cpm" throughout the chromatogram; the only reproducible non-MTX-derived peak was a small one at the front (likely ^3H_2O). Transport inhibition was performed in RPMI 1640 containing 10% horse serum and 25 mM HEPES-NaOH, pH 7.5, as previously described [43]. Inhibitory potency was assessed by pre-mixing [^{3}H]MTX with five graded concentrations of analog in 50 μl, such that when diluted to 250 μl with cells the final [^{3}H]MTX concentration was 2 μM (2 μCi/ml) and the analog concentration was as required. Uptake was initiated by addition of 200 μl of cells at $\approx 2.5 \times 10^7$ cells/ml and 2 aliquots (100 μl) were removed to iced saline and processed at 5 min. Adventitious [^{3}H]MTX binding was determined at 0° by adding 200 μl of cells to 25 μl of PBS in a tube and cooling to 0° for ≥5 min; following addition of 25 μl of [^{3}H]MTX to achieve a final concentration of 2 μM, 2 aliquots (100 μl) were immediately removed to iced saline and processed. Controls within each experiment showed that [^{3}H]MTX uptake in the absence of analog was linear for 5 min; control uptake was typically 12 pmol/10^7 cells/5 min. IC_{50} values were determined as described above. Note that competition by unlabeled MTX was not assayed because addition of unlabeled competitor MTX causes both an increase in MTX concentration and a decrease in specific radioactivity thus complicating analysis.

2.6. HPLC methods

Analytical C_{18} reversed-phase (0.4 × 25 cm; Rainin Microsorb, 5 μm) HPLC was performed at 25° as described [44]. For MTX (t_r, ≈31.6 min) and 7-OH-MTX (t_r, ≈35.2 min) the gradient was 4–13% ACN in 0.1 M Na-acetate, pH 5.5 over 41 min at 1 ml/min. CH-1504 and its analogs did not elute under these conditions; the gradient was adjusted to 4–20% ACN.

2.7. Cell culture and growth inhibition

The human T-lymphoblastic leukemia cell line CCRF-CEM [45] was cultured as described [46] and verified to be Mycoplasma-negative (Mycoplasma Plus PCR primers, Stratagene, La Jolla, CA). Growth inhibition of CCRF-CEM by continuous (120 h) drug exposure was assayed as described [46,47]. EC_{50} values (drug concentration effective at inhibiting cell growth by 50% relative to untreated control) were interpolated from plots of percent growth relative to a solvent-treated control culture versus the logarithm of drug concentration by the same method as for IC_{50} values (above).

3. Results

3.1. Metabolism of CH-1504 and analogs by FPGS

CH-1504 and its analogs (Fig. 2) are designed to be metabolism-blocked, including lacking human FPGS substrate activity because of substitution of 4′-methylene-Glu for the glutamate found in ''classical'' antifolates [16]. To verify this design aspect, substrate activity of the analogs with recombinant human cytosolic FPGS was evaluated. Substrate activity of the CHL-analogs (100 μM), with the exception of CHL-003, was at the limit of detection (≤4% activity relative to AMT), and thus the analogs are considered non-substrates for FPGS. CHL-003 possessed weak FPGS substrate activity (11% that of AMT). CHL-003 was retested at 310 μM and showed activity 13% that of AMT suggesting a K_m <100 μM and a V_{max} only 10–15% that of AMT. Analytical data on CHL-003 (Section 2) are consistent with its structure; the apparent FPGS substrate activity of this analog is currently unexplained. Because analogs could lack substrate activity but still be potent FPGS inhibitors, inhibition of substrate activity of 40 μM AMT by 100 μM analog (a concentration likely too high to be achievable in cells) was assessed. All CH-1504 analogs were only very weakly inhibitory ($IC_{50} \geq 100$ μM) to human FPGS.

3.2. Metabolism of CH-1504 and analogs by AO

The 5,8-dideaza-pyrazine ring of CH-1504 was designed to preclude 7-hydroxylation by AO. To verify that clinical grade CH-1504 and its analogs were blocked for this metabolism, their substrate activity for rabbit liver AO was tested by spectral and HPLC assays. As reported [17,20], MTX is an active substrate for AO. As assayed, MTX had a reaction $t_{1/2}$ of ≈2 min. Based on absorbance change, the reaction with MTX proceeded to ≈90% completion in 50 min, consistent with 87% completion by HPLC analysis. In contrast, no conversion of CH-1504 or any analog was observed at up to 45 min reaction times using either assay, thus all CH-1504 analogs are metabolism-blocked with respect to hydroxylation by AO.

3.3. Metabolism of CH-1504 and analogs by bacterial CPG2

Natural folates and many antifolates, including MTX [48], can be inactivated in gut by removal of the glutamate moiety intrinsic to their structure by bacterial enzymes secreted into the lumen that have substrate specificity similar to bacterial carboxypeptidase G2 (CPG2). Glutamate removal also interferes with enterohepatic circulation of folates/antifolates. CH-1504 contains 4′-methylene-Glu which is resistant to CPG2 cleavage thus blocking a third route of metabolism [16]. The ability of the well-characterized [34,49] CPG2 from *Pseudomonas* sp. strain RS-216 to remove the terminal amino acid moiety from MTX, a known substrate for this enzyme [34], and the CH-1504 analogs was compared. Spectral and HPLC assays (Section 2) were employed. As assayed, the spectral change catalyzed by CPG2 with MTX was complete in <10 min ($t_{1/2}$ ≈2 min), although the reaction was continued for 30 min. The spectrum (220–370 nm) at 10 min (not shown) was identical to that of standard 2,4-diamino-10-methylpteroic acid (DAMPA), the product of MTX hydrolysis. HPLC analysis of the reaction mixture +CPG2 at 30 min showed <1% MTX remaining and the appearance of a late eluting peak, which is presumably DAMPA. No spectral change occurred over 30 min −CPG2 and only MTX was observed by HPLC. In contrast, with 10-fold higher CPG2 and 60 min incubation, neither CH1504 nor any of its analogs exhibited any significant change in UV–visible spectrum. HPLC analysis of the CPG2-containing reactions showed no additional peaks and >93% (by area; 93–110% range) of the absorbance was recovered at the same retention as parent drug with no later eluting peak equivalent to a des(4′-methylene-Glu) product. Recovery of MTX, CH1504, and all analogs in reactions −CPG2 was >90% (by area; 90–106% range). Removal of the terminal 4′-methylene-Glu would produce a substituted benzoate in cases where a benzoyl moiety is present and thus, similar to MTX, a spectral change would be expected if hydrolysis occurred. In addition, because a reversed-phase HPLC column was used for analysis, it is likely that the large change in polarity caused by removal of 4′-methylene-Glu from the analogs would alter retention. Based on the absence of spectral change and alteration in HPLC retention, it is concluded that no hydrolysis occurred and that none of the CH-1504 analogs are CPG2 substrates.

3.4. Human DHFR inhibition

The above results show that CH-1504 and its analogs are metabolism-blocked, with the exception of weak FPGS activity of CHL-003. CH-1504 (then known as AA-243 or Mobiletrex) was reported to be a potent DHFR inhibitor [16]. Newly synthesized clinical grade CH-1504 had the same high DHFR inhibitor potency as AA-243, but both were ≈2.5-fold less potent than MTX (Table 1). However, both AA-243 and CH-1504 are diastereomeric mixtures of analogs containing L- and D-4′-methylene-Glu, while MTX contains only L-glutamic acid (Glu). Substitution of a D-congener alone in MTX [50] and AA-243 [31] markedly decreases DHFR inhibitory potency. Thus, the potencies of L-AA-243 and L-CH-1504 are very similar to that of L-MTX. CHL-002, -004, and -005 have DHFR inhibitory potency similar to CH-1504. These analogs again share the issue of chirality in the 4′-methylene-Glu moiety so potency of the L-isomers could be up to 2-fold higher. CHL-001 and -003 are significantly less potent DHFR inhibitors.

Table 1 – Inhibition by MTX and other folate analogs of human folate-dependent enzymes. IC_{50} values for each enzyme were determined as described in Section 2.

Drug	IC_{50}[a]				
	DHFR (nM)	TMPS (μM)	GARFT (μM)	AICARFT (μM)	MTHFR (μM)
MTX	0.45 ± 0.1 (*n* = 4)	47 ± 11 (*n* = 5)	>50	>50	33 ± 10 (*n* = 4)
MTX-γ-Glu_2	ND[b]	ND[b]	ND[b]	ND[b]	2.2 ± 0.3 (*n* = 2)
MTX-γ-Glu_4	ND[b]	ND[b]	ND[b]	ND[b]	0.7 ± 0.1 (*n* = 2)
AA-243	1.25 ± 0.05 (*n* = 2)	ND[b]	>50	>50	ND[b]
CH-1504	1.1 ± 0 (*n* = 2)	8.0 ± 0 (*n* = 2)	>50	>50	1.6 ± 0.2 (*n* = 3)
CH-001	73 ± 8 (*n* = 2)	>>50 (*n* = 2)	>50	>50	>50
CH-002	2.7 ± 0.7 (*n* = 3)	4.9 ±0 (*n* = 2)	>50	>50	1.8 ± 0.2 (*n* = 2)
CH-003	12.5 ± 1.5 (*n* = 2)	>50 (*n* = 2)	>50	>50	>48 (*n* = 3)
CH-004	1.65 ± 0.05 (*n* = 2)	4.8 ± 0.1 (*n* = 2)	>50	>50	1.2 ± 0.2 (*n* = 2)
CH-005	1.8 ± 0.3 (*n* = 3)	>50 (*n* = 2)	>50	>50	3.5 ± 0.6 (*n* = 2)

[a] Values are average ± S.D. for $n > 2$. For $n = 2$ values are average ± range.
[b] ND = not determined.

3.5. *Inhibition of RFC-mediated uptake of [^{3}H]MTX by human CCRF-CEM cells*

Two transport systems mediate uptake of reduced folates and antifolates in human cells: the reduced folate carrier (RFC; SLC19A1) and the folate binding protein family [51]. The RFC is the most widely distributed and is generally considered the primary transport mechanism [51]. CCRF-CEM cells express only RFC so the interaction of this carrier with CH-1504 and its analogs can be measured by their potency as inhibitors of [^{3}H]MTX influx (Table 2). The known "good" RFC substrate AMT, the 10-desmethyl analog of MTX, inhibits with high potency, as expected [43]. Under these same conditions, folic acid (a poor substrate for the RFC) was previously shown to have an IC_{50} of 19 μM and to plateau at 60–70% inhibition [43], unlike MTX or CH-1504 or its analogs. Clinical grade CH-1504, its progenitor AA-243, CHL-002, and CHL-005 are essentially as potent as AMT. CHL-003 and CHL-004 have 2.5–3-fold higher apparent affinity for the RFC than does AMT or CH-1504. Since each compound is a D,L-racemate (above) and since D-MTX at ≤10 μM has no effect on transport of 1 μM L-[^{3}H]MTX [50], their L-enantiomers may have up to a 2-fold higher affinity for the human RFC than does AMT. CHL-001 alone displayed poor inhibitory potency for the RFC; assuming that this reflects its transport, these data and its poor DHFR inhibitory potency (Table 1) suggest that 4-desamino-4-methyl substitution is not promising.

Table 2 – Inhibitory potency of antifolates against CCRF-CEM human leukemia cell RFC activity. Inhibitory potency of aminopterin (AMT), AA-243 (Mobiletrex), CH-1504, CHL-001 through CHL-005 against CCRF-CEM RFC was assessed by inhibition of uptake of 2 μM [^{3}H]MTX by intact cells. Experiments were performed in two sets at different times (upper and lower halves of table). The values for AMT show the reproducibility of the assay over time. All values are average ± range for duplicate determinations. Note that because L-[^{3}H]MTX is used to assess uptake, competition by unlabeled L-MTX was not assayed. Addition of unlabeled L-MTX, as in a typical competition experiment, causes both an increase in MTX concentration and a decrease in specific radioactivity and thus complicates analysis. Based on McGuire et al. [42], however, the apparent IC_{50} for MTX would be ≈9 μM.

Inhibitor	IC_{50} (μM)
Aminopterin	2.7 ± 0
AA-243	2.9 ± 0.5
CH-1504	2.9 ± 0.1
CHL-003	1.0 ± 0.2
CHL-004	1.2 ± 0.4
Aminopterin	1.9 ± 0.1
CHL-001	8.5 ± 0.9
CHL-002	1.6 ± 0.2
CHL-005	1.8 ± 0.4

3.6. *Inhibition of other relevant human folate-dependent enzymes*

Thymidylate synthase (TMPS) and two folate-dependent enzymes involved in *de novo* purine synthesis, GAR formyltransferase (GARFT) and AICAR formyltransferase (AICARFT), are targets of several clinical and preclinical antifolates. Folate analogs are generally either highly potent inhibitors of these enzymes as monoglutamates or are poor inhibitors as monoglutamates and are only effective once polyglutamylated [52]. Since none of the CH-1504 analogs, with the possible exception of CHL-003, are FPGS substrates (above), if the analogs *per se* are not potent inhibitors of isolated human folate-dependent enzymes, then those enzymes are unlikely to be targets in whole cells. Inhibition of human TMPS by all compounds tested was very weak (μM) compared to their inhibition of DHFR (Table 1; DHFR values are nM). Likewise none of the compounds inhibited AICARFT or GARFT activity by >50% (Table 1) even at 50 μM. These data suggest that TMPS, GARFT, and AICARFT would not be targets of any of these analogs in intact cells. MTHFR activity has been associated with toxicity during MTX therapy of RA [53]. MTX itself is a poor MTHFR inhibitor, but poly(γ-glutamylation) increases potency; the triglutamate and the pentaglutamate are 15- and 47-fold more potent, respectively, than MTX (Table 1). CHL-001 and -003 are very weak inhibitors of MTHFR, while CH-1504 and the other analogs exhibit low μM potency (Table 1).

Table 3 – Antifolate diastereomer inhibitory potency against CCRF-CEM human leukemia cell DHFR, RFC activity, and cell growth. Inhibitory potencies of methotrexate (L-MTX), D-MTX, aminopterin (L-AMT), DL-CH-1504, Peak 1, and Peak 2 were determined. For DHFR, RFC, and MTHFR, values are average ± range for $n = 2$, and average ± S.D. for $n > 2$. A separate preparation of DHFR was used to perform this set of studies and those of Table 1. Each DHFR preparation can have slightly different inhibitor sensitivity, which is normalized for by including MTX as a positive control. The values of other analogs relative to MTX will always be the same; thus, DL-CH-1504 is about 2-fold less potent than MTX in both Tables 1 and 3. Growth inhibitory potency of methotrexate (MTX), DL-CH-1504, Peak 1, and Peak 2 against CCRF-CEM human leukemia cells was measured following continuous (120 h) exposure. Values for growth inhibition are average ± S.D. for triplicate determinations, except for L-MTX ($n = 6$) and DL-CH-1504 ($n = 5$).

Inhibitor	IC_{50}			CCRF-CEM growth inhibition EC_{50} (nM)
	DHFR (nM)	RFC (μM)	MTHFR (μM)	
L-MTX	1.1 ± 0	ND[a]	33 ± 10	14.0 ± 0.8
D-MTX	ND[b]	49 ± 1	ND[a]	ND[b]
L-AMT	ND[a]	1.6 ± 0.1	ND[a]	ND[b]
DL-CH-1504	2.6 ± 0.2	1.8 ± 0.1	1.6 ± 0.2	7.7 ± 0.9
Peak 1	1.3 ± 0.1	1.1 ± 0	1.1 ± 0.1	5.2 ± 0.7
Peak 2	7.6 ± 0.1	7.0 ± 0.6	2.8 ±0.2	68 ± 20

[a] ND = not determined.
[b] Previous data [50] shows that for DHFR inhibition, D-MTX IC_{50} = 5.6 nM, when L-MTX EC_{50} = 0.6 nM. For CCRF-CEM growth inhibition, D-MTX EC_{50} = 535 nM, when L-MTX EC_{50} = 16 nM; L-AMT EC_{50} = 2.9 nM, when L-MTX EC_{50} = 12 nM [63].

3.7. Activity of CH-1504 diastereomers

As noted above, clinical grade CH-1504 is a diastereomeric mixture containing L- or D-4′-methylene-Glu. The striking biochemical properties and intriguing initial clinical data for CH-1504 [27] prompted an examination of the properties of its individual diastereomers, which were resolved by preparative chiral chromatography (Section 2). Since racemic CH-1504 was metabolism-blocked and did not significantly interact with TMPS, GARFT, or AICARFT (above), the individual diastereomers were evaluated only for interaction with DHFR, RFC, and MTHFR, and for growth inhibitory potency. With respect to DHFR inhibition, Peak 1 was more potent than racemic CH-1504 and had potency similar to MTX, while Peak 2 was 6-fold less potent (Table 3). This underestimates the difference in potency, however, because the slope of the concentration–effect curve of Peak 1 is steep and similar to that of MTX, while the slope for Peak 2 is more shallow (not shown) indicating the relative concentration required to achieve >98% DHFR inhibition, as is required for a biological effect [54], will be higher than the 6-fold difference seen at the IC_{50} level. Peak 1 is more potent than racemic CH-1504 (and similar to AMT) as an inhibitor of [^{3}H]MTX uptake by the RFC (Table 3), suggesting that it also has high affinity for the RFC. Peak 2 has 6-fold lower apparent affinity for the RFC than does Peak 1. The MTX analog containing D-glutamic acid (D-MTX) has a relatively poor affinity compared to the K_t for L-MTX (3 μM; [43]) in this cell line. Peak 1 was also slightly more inhibitory to MTHFR than was Peak 2, but the difference was relatively small. The disparate characteristics of Peaks 1 and 2 are reflected in their growth inhibitory properties against CCRF-CEM human T-cell leukemia cells in continuous exposure. Peak 1 is 8-fold more potent than Peak 2 as a growth inhibitor.

Table 4 – Growth inhibitory potency of antifolates against CCRF-CEM human leukemia cells. Growth inhibitory potency was measured as described in Section 2 following continuous (120 h) exposure. Values for growth inhibition are average ± range for duplicate determinations and average ± S.D. for $n \geq 3$ determinations.

Inhibitor	CCRF-CEM growth inhibition EC_{50} (nM)
L-MTX	14.0 ± 0.8 ($n = 6$)
DL-CH-1504	7.7 ± 0.9 ($n = 5$)
CHL-001	5200 ± 600 ($n = 2$)
CHL-002	15 ± 2 ($n = 2$)
CHL-003	44 ± 9 ($n = 2$)
CHL-004	14 ± 1 ($n = 2$)
CHL-005	4.1 ± 0.7 ($n = 2$)

3.8. Growth inhibitory potency of CH-1504 analogs

The potency of CHL-001 through CHL-005 as growth inhibitors of CCRF-CEM cells was also evaluated (Table 4). In this group, only CHL-001 displayed low potency in accord with its poorer apparent transport and weaker inhibition of DHFR. CHL-002, -003, -004, and -005 were in the same range of potency as MTX and CH-1504 and broadly reflected their respective potencies as DHFR inhibitors and affinities for the RFC. CHL-005 was about 2-fold more potent than its parent CH-1504 and represents a promising new lead for this class of analog.

4. Discussion

CH-1504, a new antifolate for the treatment of RA [26,28], is the clinical form of 4-amino-4-deoxy-5,8,10-trideazapteroyl-DL-4′-methylene-Glu (AA-243 or Mobiletrex) that was rationally designed by Nair et al. [16] to be blocked in pathways of metabolism common to MTX, the current standard of care in RA [2]. The design posited that metabolism of MTX was responsible for all or part of the toxicity of MTX at the low doses used in RA therapy and that elimination of such metabolism would lead to an increased therapeutic benefit or similar benefit at lower toxicity, and thus lead to better compliance. It was critical to confirm that clinical grade CH-

1504 was metabolism-blocked and also possessed the biochemical and biological properties of the original compound. The data (Tables 1 and 2; text) show that CH-1504 is not a substrate for poly(γ-glutamylation) by FPGS, for hydroxylation by AO, or for deglutamylation by bacterial CPG2-like peptidases active in the intestinal lumen and is thus metabolism-blocked. CH-1504 is a DHFR inhibitor with potency identical to AA-243 and only 2-fold less potent than MTX itself; the latter difference is primarily a result of CH-1504 being a diastereomeric mixture (*vide infra*). As a monoglutamate, CH-1504, identical to AA-243, is a very poor inhibitor of other key enzymes in folate metabolism whose inhibition could impact its mechanism of action, viz. TMPS, GARFT, and AICARFT (Table 1). Since CH-1504, unlike MTX [11], cannot be polyglutamylated to increase its potency against these secondary targets, these data show that direct inhibition of *de novo* purine synthesis (either at GARFT or AICARFT) or thymidylate synthesis, will not play a role in the mechanism of action of CH-1504 in intact cells. Thus, CH-1504 is a pure DHFR inhibitor. It was previously shown [16] that CH-1504 (AA-243) uses the RFC for uptake, at least in human CCRF-CEM cells, and substitution by 4′-methylene-Glu was indirectly inferred to enhance RFC affinity [55]. Since radiolabelled CH-1504 is not yet available, measurement of relative affinities of AA-243 and CH-1504 for the RFC was estimated herein from their ability to compete with [^{3}H]MTX uptake (Table 2). CH-1504 was equipotent with AA-243 and both were similar in potency to AMT, a recognized good substrate for the RFC (diastereomers of CH-1504 are considered below). While informative, such competition experiments have limitations, principally not establishing overall transport efficiency ($V_{max,t}/K_t$). To overcome these limitations, radiolabeled CH-1504 will be prepared and its transport kinetics measured directly. The substrate activity of CH-1504, its isomers, and its analogs for the newly described proton-coupled folate transporter (PCFT; [56]) is of obvious interest because of its role in intestinal folate uptake and the fact that the oral route of administration of anti-RA antifolates is preferred. Characterization of transport by the PCFT using radiolabeled drugs will also be the subject of future studies.

MTHFR activity expression has been associated with toxicity during MTX therapy of RA [53]. MTX itself had very low human MTHFR inhibitory potency (Table 1) relative to its DHFR inhibitory potency, a result similar to that reported for rat brain [57] and purified human liver [58] MTHFRs. Polyglutamylation enhances the kinetic efficiency (V_{max}/K_m) of 5,10-CH_2-H_4PteGlu$_n$ substrates for MTHFR [59] and potentiates the inhibitory potency of MTX for several folate-dependent enzymes [11]. Thus, the effect of MTX polyglutamylation was also of interest. Addition of as few as two γ-glutamates to MTX increased its potency against human MTHFR significantly and addition of four γ-glutamates increased potency nearly 50-fold (Table 1). These lengths are important because MTX-(γ-Glu$_2$) is the shortest chain length that is highly retained by cells [60] in the absence of extracellular drug and MTX-(γ-Glu$_4$) is a prominent longer-chain MTX polyglutamate synthesized by human cells after low-dose MTX treatment [10,61]. These inhibition data with cloned wild-type (C677 and A1298) human MTHFR [41] are similar to those reported for human liver MTHFR of unknown genotype with MTX and MTX-(γ-Glu$_4$) [58]. CH-1504 and even the most potent 4′-methylene-Glu-containing analogs inhibit MTHFR in the low μM range, much weaker than their DHFR inhibition potency. Although the intrinsic potencies against MTHFR of CH-1504 (and several analogs) are not dramatically weaker than MTX-(γ-Glu$_4$), polyglutamylation confers properties on MTX that increase the possibility of MTHFR inhibition in cells. Unmetabolized intracellular MTX and nonpolyglutamylatable antifolates like CH-1504 efflux from cells rapidly as plasma levels fall. However, MTX polyglutamates efflux poorly and turn over slowly and are therefore retained within cells for long periods of time; thus their inhibitory effects on MTHFR (and other targets) are prolonged.

CH-1504 is synthesized with DL-4′-methylene-Glu and thus contains two diastereomers. Literature data show that antifolates containing D-amino acids are much less potent than those containing their L-congeners (cf. MTX [50]). To investigate their properties, the CH-1504 diastereomers were resolved by preparative chiral chromatography. Peak 1 was more potent than Peak 2 as a DHFR inhibitor, as a competitor of [^{3}H]MTX uptake, and as a growth inhibitor; it had properties similar to those of MTX, except that it is metabolism-blocked. Nair et al. previously briefly reported that the L-4′-methylene-Glu-containing diastereomer was the more potent [31] and thus Peak 1 corresponds to that diastereomer. Peak 2 (D-isomer), however, retained significant activity in each assay. Note that each diastereomer is contaminated with ≈3% of the opposite isomer (Section 2). Three percent of Peak 2 in Peak 1 would make only an insignificant change in the IC_{50} of Peak 1 for DHFR. Three percent Peak 1 in Peak 2 probably contributes some inhibition, but cannot account for a 7.7 nM IC_{50}, thus the D-isomer *per se* must have significant potency (although still ≥6-fold less potent than L-CH-1504). Although the less active isomer could be considered helpful because its mechanism of action is the same or could be considered innocuous because of its lower potency, the presence of a second isomer could complicate use of CH-1504 from a pharmacokinetic perspective [62]. Since the effect of the less active diastereomer would have to be studied extensively in vivo to determine whether it was detrimental, it seems, as in the case of most drugs, that the single most active isomer is preferred.

CH-1504 represents the first generation of metabolism-blocked anti-RA agents containing 4′-methylene-Glu. Second generation CH-1504 analogs (Fig. 2) contain alternate heterocycles and benzoyl substitutions in an attempt to enhance their biochemical properties, while maintaining their block in metabolism. Within this initial group are represented structures that could potentially increase DHFR inhibitory potency and/or increase transport; effects of these changes on pharmacokinetics are unknown, however. To simplify synthesis for initial characterization, each analog was prepared as a mixture of diastereomers and thus the activity of the most active component could be up to 2-fold higher. As with CH-1504, each analog was blocked with respect to AO and CPG2 activity, as determined by two analytical methods. Each analog, with the exception of CHL-003, was also devoid of human FPGS substrate activity. The low FPGS substrate activity of CHL-003 is not understood at present. None of the

analogs significantly inhibited human TMPS, GARFT, or AICARFT (Table 1). CH-002, -004, and -005 were all potent DHFR inhibitors, similar to CH-1504. CH-002, -003, -004, and -005 all exhibited affinity for the RFC at least as high as CH-1504 (Table 2), although whether this translates into better overall transport cannot be stated (see above); only CH-001 had poor apparent affinity for the RFC. A correlation of DHFR potency, RFC affinity, and growth inhibitory potency suggests that CHL-005, the 5-aza analog of CH-1504, might be an attractive candidate for further preclinical evaluation. If diastereomers of CH-005 behave analogously to those of CH-1504 (above), then the properties of L-CH-005 could be up to 2-fold more positive. The difficulty in choosing among candidate analogs, however, is exemplified by CH-003, which is a relatively poor DHFR inhibitor but exhibited the highest apparent affinity for the RFC; again the L-isomer would likely have higher affinity for both DHFR and the RFC. Further in vivo studies will be required to validate these biochemical data in model systems for RA.

The current studies do not directly address the mechanism(s) of action of CH-1504 and its analogs in clinical RA therapy. However, since CH-1504 and its active analogs are potent DHFR inhibitors similar to MTX, but do not directly inhibit other relevant folate-dependent enzymes, it is likely their effects on folate metabolism are mediated by depletion of the reduced folate pool. Because virtually all of the currently proposed mechanisms for MTX action in RA (see Section 1) can be induced by reduced folate depletion, the clinical mechanism(s) of MTX and CH-1504 are likely similar or identical. The advantage of CH-1504 is not in a different mechanism of anti-RA action, but in its potential for reduced toxicity.

In summary, biochemical characterization of heterocycle and/or benzoyl moiety modified analogs of CH-1504, a prototypical metabolism-blocked inhibitor of DHFR, suggests that several modifications lead to novel compounds with potential anti-RA activity that should be evaluated in preclinical animal models. CH-1504 itself is a diastereomeric mixture and the data show that one isomer is significantly superior with respect to several key determinants of action suggesting that this single isomer is preferred for further clinical development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.12.020.

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