Articles

Design and Synthesis of Dihydrofolate Reductase Inhibitors Encompassing a Bridging Ester Group. Evaluation in a Mouse Colitis Model

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Crohn's disease is a chronic inflammatory bowel disease characterized by inflammation of both the small and large intestines. Methotrexate (MTX), a classical dihydrofolate reductase (DHFR) inhibitor, has been used as a therapeutic agent in the treatment of patients with Crohn's disease in recent years. We sought to develop antifolates similar in structure to MTX that would be effective in reducing inflammation in a mouse disease model of colitis. Four classical DHFR inhibitors encompassing ester bridges in the central parts of the molecules were synthesized. These antifolates were efficient inhibitors of the DHFR enzyme derived from rat. They were also tested in vitro for their ability to inhibit induced proliferation of lymphocytes from mouse spleen. Inhibition of cell proliferation was achieved only in the micromolar range, whereas MTX was effective at low nanomolar concentrations. One of the DHFR inhibitors (**1**), with an IC_{50} value for rlDHFR approximately 8 times higher than that of methotrexate, was selected for in vivo experiments in an experimental colitis model in mice. This compound demonstrated a clear antiinflammatory effect after topical administration, comparable to the effect achieved with the glucocorticoid budesonide. Three parameters were evaluated in this model: myeloperoxidase activity, colon weight, and inflammation scoring. A favorable in vivo effect of compound **1** (15 mg/(kg·day)) was observed in all three inflammatory parameters. However, the results cannot be explained fully by DHFR inhibition or by inhibition of lymphocyte cell proliferation, suggesting that other yet unidentified mechanisms enable reduction of inflammation in the colitis model. The mechanism of action of methotrexate analogues encompassing a bridging ester group is not well understood in vivo but seems to lend itself well to further development of similar compounds.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD). CD affects both the small and large intestines, whereas UC only involves the colon. The etiology of IBD is unclear, and no curative treatments are currently available. Pharmacological treatments can induce remissions, but most often these are followed by relapses. The standard treatments for IBD have been aminosalicylates and glucocorticoids in the past decades. Sulfasalazine and 5-aminosalicylates are used in the management of mild to moderate disease, whereas the glucocorticoids remain the primary therapy for patients suffering from moderate to severe disease. Of these therapies, the glucocorticoids are less effective in maintaining remission in UC patients and are in addition often associated with severe side effects in long-term treatment. The fact that nearly all drugs absorbed from the bowel pass the liver before entering systemic circulation provides an opportunity to improve the therapeutic index of drugs aimed for topical therapy of the intestinal mucosa. Thus, budesonide (Figure 1), designed by one of us to undergo an efficient first-pass metabolism in the liver, is now used in the clinic. When delivered in an appropriate pharmaceutical formulation, thereby reaching the inflamed gut segment, a significantly improved ratio of the designed topical actions to the adverse systemic action is achieved.[1] In recent years, immunosuppressive agents, e.g., azathioprine, 6-mercaptopurine, cyclosporin A, and methotrexate (Figure 2) as well as monoclonal antibodies against TNFα,[2] have found an increasing and widespread use for the treatment of patients with IBD.[3] The first reports on methotrexate (MTX), a potent inhibitor of dihydrofolate reductase, and its potential role in IBD therapy appeared in 1989.[4] During the 1990s, further support for a beneficial role of MTX was reported, and more recently, it was demonstrated that patients with Crohn's disease who entered remission after a low dose of MTX also seemed to maintain remission after long-term therapy.[5−9] MTX has also

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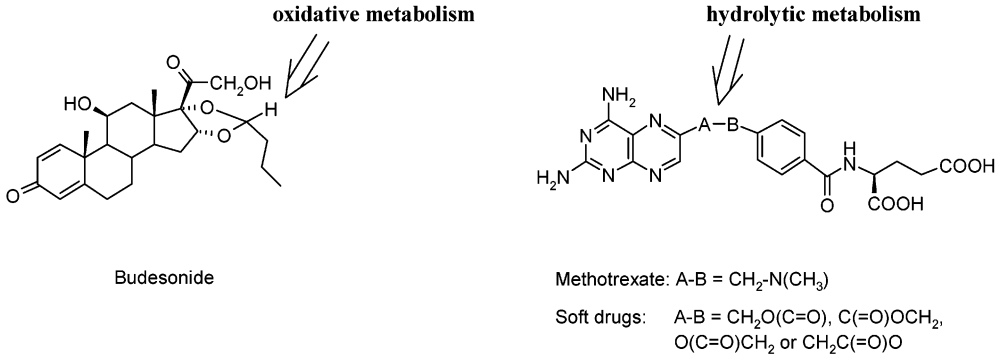


Figure 1.

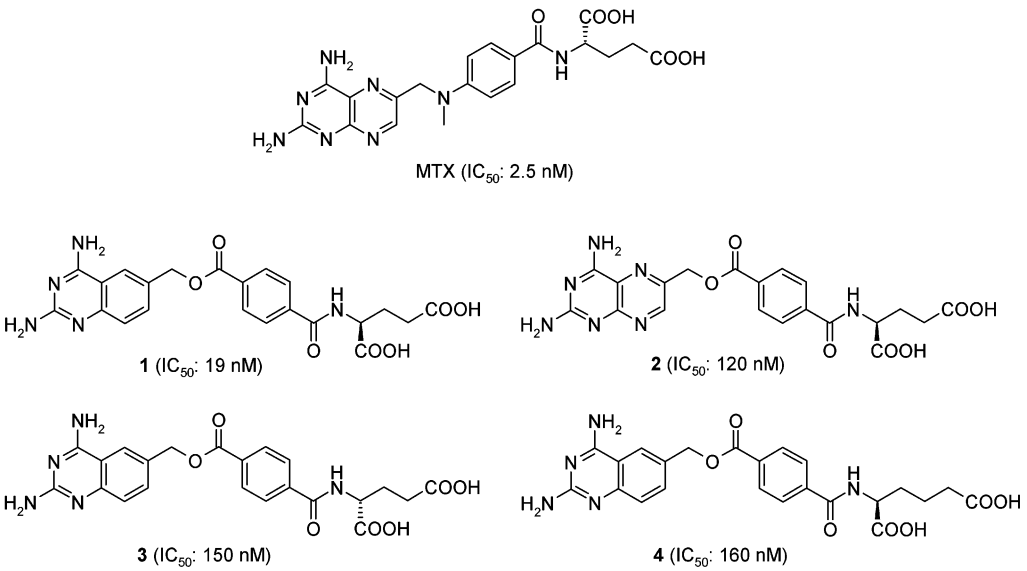


Figure 2. rlDHFR inhibition (IC_{50}) indicated for each substance.

been demonstrated to decrease the need for glucocorticoids in glucocorticoid-dependent CD.[6,10]

Our long-term perspective is to develop soft drug analogues of antifolates that, like budesonide, are efficiently transformed locally in the intestinal mucosa, in serum or in the liver, to nontoxic metabolites after having exerted their antiinflammatory effects in the intestinal tissues. While budesonide undergoes an efficient oxidative deactivation in liver mediated by the cytochrome P450 system[1] (Figure 1), other soft drugs, e.g., of cyclosporin A, are designed to undergo deactivation mediated by esterases.[11] We postulated that the methylenamino bridge in MTX could constitute a suitable site for a diversity of structural manipulations including the incorporation of an appropriate ester feature to provide good substrates for the endogenous esterases while still retaining the inhibitory activity of dihydrofolate reductase (DHFR) (Figure 1).

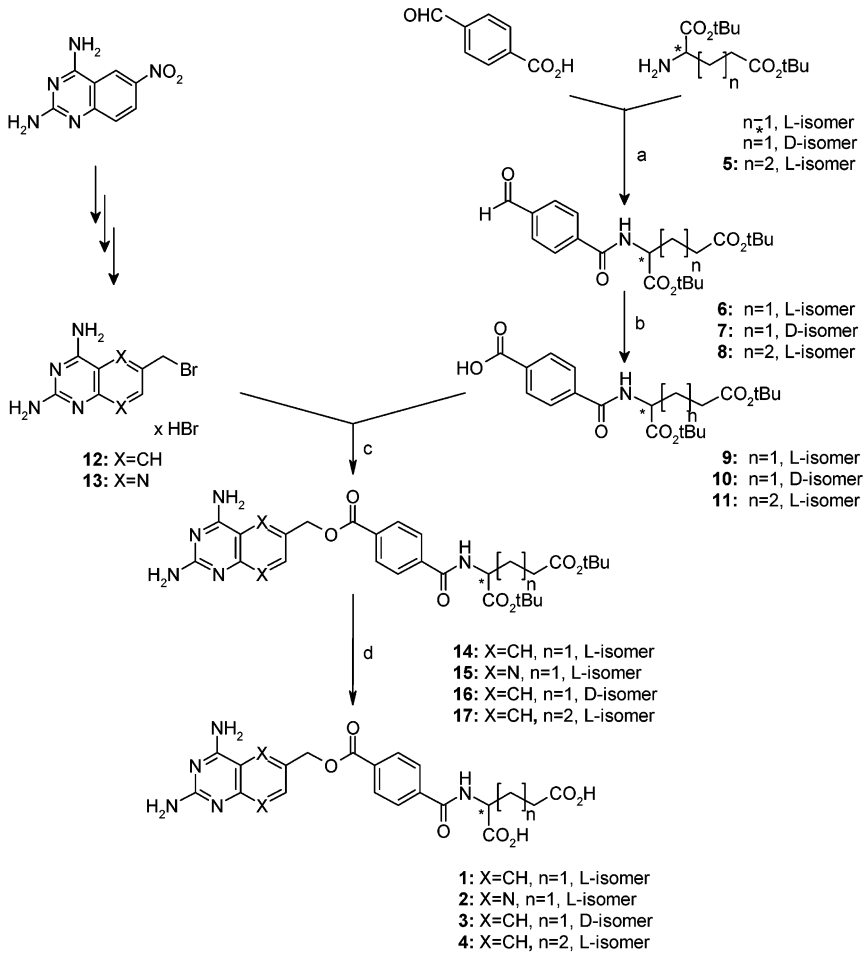
As a first step toward soft drug analogues of MTX, it was essential to probe the tolerance for structural alterations in (a) the heterocyclic core[12] and (b) the hydrophilic side chain of the new ester-containing antifolates. Would (a) the in vitro inhibitory activity and the ability to inhibit cell proliferation remain and (b) the in vivo activity in complex animal models remain after the methyleneamino group in the classical antifolates was substituted for an ester group? To address these issues, we examined a small set of short-bridge, metabolically stable ester analogues and evaluated the impact of an ester-containing bridge on the bioactivity in vivo. We previously identified **1** and **2**, consisting of a quinazoline and a pteridine core, respectively, as inhibitors of DHFR.[12]

The most potent DHFR inhibitor (**1**) was found to exert an impressive activity after local administration in an experimental inflammatory bowel disease model in mice. This effect was similar to that obtained with systemic administration of a moderate dose of the potent glucocorticoid budesonide.

Results

Syntheses. Compounds **1**−**4** were synthesized with the reaction sequence outlined in Scheme 1. We have previously prepared compounds **1** and **2** by an alternative procedure that was partly based on solid-phase chemistry.[12] Di-*tert*-butyl L-α-aminoadipate[13] (**5**) was coupled to 4-formylbenzoic acid using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine, affording **8**. Compounds **6**[14] and **7**[15] were synthesized in a similar manner starting from the commercially available di-*tert*-butyl esters of L- and D-glutamic acid hydrochloride, respectively. The aldehydes were oxidized immediately after purification to the corresponding carboxylic acids **9**−**11**, using sodium chlorite in *tert*-butyl alcohol with 2-methyl-2-butene as scavenger.[16−18] 2,4-Diaminoquinazoline-6-methanol[19] was subjected to 30% hydrobromic acid in glacial acetic acid,[20] providing compound **12**.[12]

Scheme 1[a]



[a] (a) PyBOP, Et_3N, or *N*,*N*-diisopropylethylamine, CH_2Cl_2; (b) $NaClO_2$, $NaHPO_4 \cdot 1H_2O$, 2-methyl-2-butene, *tert*-butyl alcohol; (c) K_2CO_3, DMF, or DMAc; (d) HCO_2H or CF_3CO_2H.

The reaction mixture was triturated with cold diethyl ether to allow for precipitation, which was easily collected under an atmosphere of nitrogen before it was dried at 40 °C in vacuo and used in the next step without further purification. The 6-(bromomethyl)-2,4-diaminopteridine hydrobromide (**13**)[12,21,22] was prepared according to the same method as for **12** starting from commercially available 2,4-diaminopteridine-6-methanol. The esters **14**−**17** were formed by nucleophilic displacement of the appropriate bromide by the benzoates **9**−**11**. The overall yield for the last two steps varied between 23% and 51%. Prolonging the reaction time or heating the reaction mixture did not seem to improve the yields. A final selective hydrolysis of the *tert*-butyl esters with formic acid or trifluoroacetic acid provided compounds **1**−**4** in good yields.

Dihydrofolate Reductase Inhibition. Compounds **1**−**4** were examined for their inhibitory activity of rat liver DHFR according to published procedures.[23] The results are presented as IC_{50} values (Figure 2). Compound **1** was found to be the best inhibitor of rlDHFR with an IC_{50} value only 8-fold higher than that of MTX. However, compounds **2**−**4** exhibited a greater than 10-fold loss of potency relative to **1**. To assess the impact of the glutamic acid residue and the methyleneamino bridge, the IC_{50} values of nine representative derivatives were included for comparison (Figure 3). As apparent from the figure, displacement of the glutamic acid portion rendered less active inhibitors.

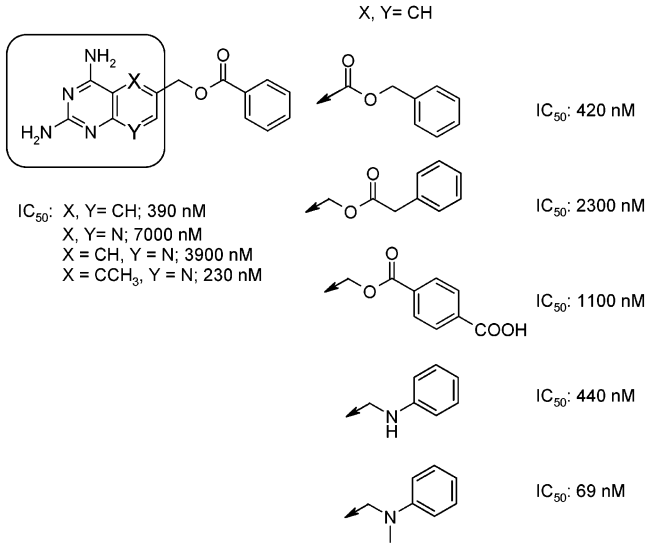


Figure 3. rlDHFR inhibition (IC_{50}) indicated for each substance.

Cell Proliferation Assay. Because MTX and compounds **1**−**4** are inhibitors of DHFR of varying potency, the ability of these compounds to inhibit mitogen-induced proliferation of mouse spleen cells (lympho-

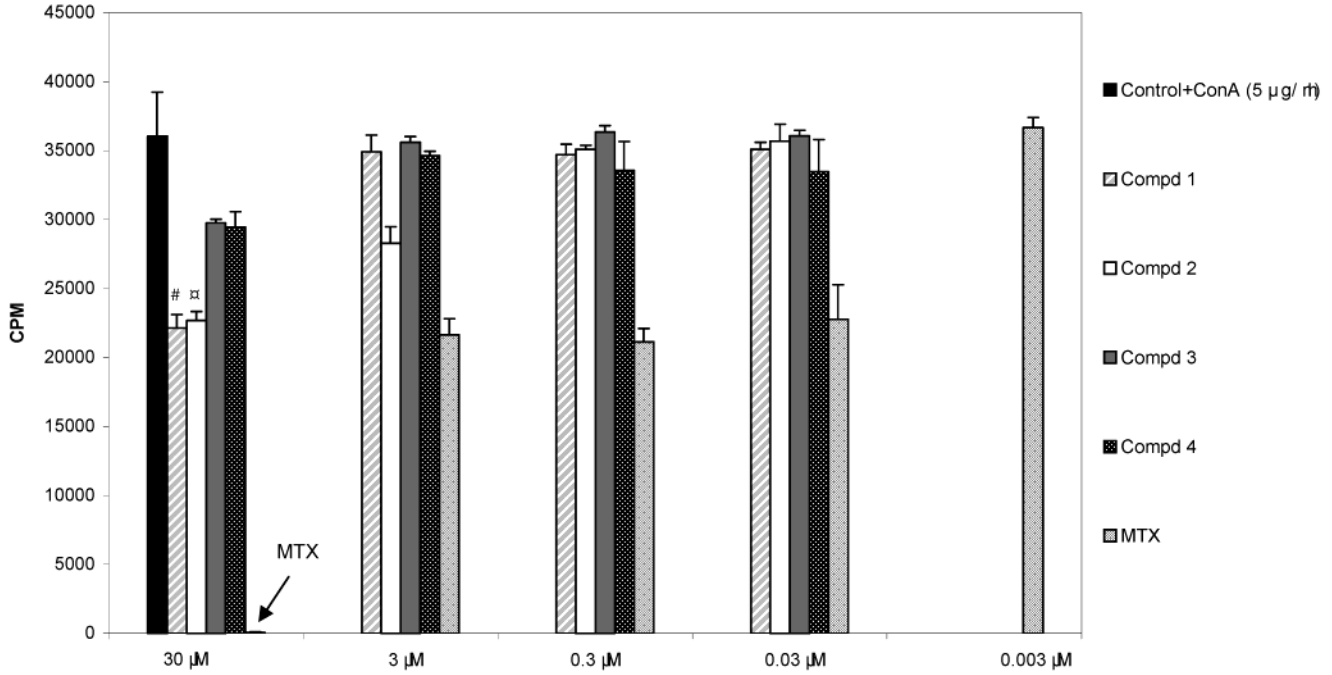


Figure 4. Effect of MTX and compounds **1**−**4** on ConA-mediated mouse spleenocyte proliferation. CPM is counts per minute. Proliferation was induced by ConA (5 μg/mL), and compounds were added at concentrations as indicated. Two days after initiation of the experiment, ^{3}H-thymidine was added and labeling was continued for 18 h. Bars indicate the mean of triplicates. Error bars indicate SD. Only MTX was tested at 3 nM. Significant difference from control group is indicated: (#) $p < 0.001$; (○) $p < 0.01$.

cytes) was compared. Inhibition of lymphocyte proliferation could explain at least part of an antiinflammatory effect in vivo. Cells were thus purified from mouse spleens and thereafter stimulated with the T-cell mitogen concanavalin A (ConA). Inhibition of proliferation with MTX and compounds **1**−**4** was evaluated at various concentrations (Figure 4). MTX inhibited proliferation at 30 nM and higher. Similar data were obtained in two independent experiments. Compound **1** suppressed cell proliferation at only the highest dose compared (30 μM), a 1000-fold higher concentration than that of MTX. Thus, the inhibition of mouse lymphocyte proliferation was not well correlated with inhibition of the enzyme DHFR.

Colitis Model in Mice. The antiinflammatory effects of compound **1** and MTX were compared in a mouse model of inflammatory bowel disease. In this model, locally instilled DNBS (2,4-dinitrobenzenesulfonic acid) is used to induce an acute inflammation of the colon. The effect of the compounds was assessed after rectal administration once daily for 7 days. As a reference compound, the potent steroidal soft drug budesonide was administered once daily by intraperitoneal injection. It should be noted that systemic administration of budesonide does not take advantage of the high first-pass metabolism obtained with this compound following oral administration. However, in this experimental setting, we wanted to ensure significant systemic glucocorticosteroid effect as a positive reference for the effect of the treatment. Previous data showed that the dose of budesonide used, 1 μg/(kg·day) given intraperitoneally, produced a pronounced systemic glucocorticosteroid effect.[24] The doses of MTX and compound **1** were chosen on the basis of earlier experiments in rodents with MTX[12,25] and the relative potency of the substances regarding DHFR inhibition.[12] Previous work also demonstrated that the maximal well-tolerated dose of MTX to mice is 0.5 mg/(kg·day), and preliminary data also indicated positive effects of this dose in the mouse colitis model (data not shown).

In the current study, it was found that the reference compound, budesonide, suppressed the inflammatory response significantly (Figure 5). However, MTX at the given dose (0.5 mg/(kg·day)) had no effect on inflammation assessed by three different methods. Compound **1** was given to mice at two different doses, 15 and 60 mg/(kg·day), to meet and even exceed the relative difference in potency between MTX and compound **1** with respect to DHFR inhibition. Interestingly, only the lower dose of compound **1** (15 mg/(kg·day)) reduced the inflammatory response. The effect of the lower dose was comparable to that of budesonide, and it also differed significantly from the inflammation in the control group of animals. In addition to the parameters measured, it was noted that the animals receiving the 15 mg/(kg·day) dose of compound **1** were in much better general condition than those receiving the higher dose. There were no obvious effects on health status of the animals when compound **1** was given to control animals.

Discussion

The underlying mechanism behind the effects of methotrexate in IBD therapy is still not fully elucidated. It has been proposed that the antiinflammatory action of methotrexate is due to accumulation of adenosine,[26] and adenosine A_2 receptor antagonists have been shown to suppress the antiinflammatory effect of MTX.[27] However, the role of adenosine as an important mediator of the antiinflammatory effect of MTX has been questioned[28] and is not yet proven in a clinical situation.[29] AICARtf (5-aminoimidazole-4-carboxamide ribonucleotide transformylase), the last enzyme in de novo purine synthesis, is inhibited by methotrexate polyglutamates and is suggested as another candidate.

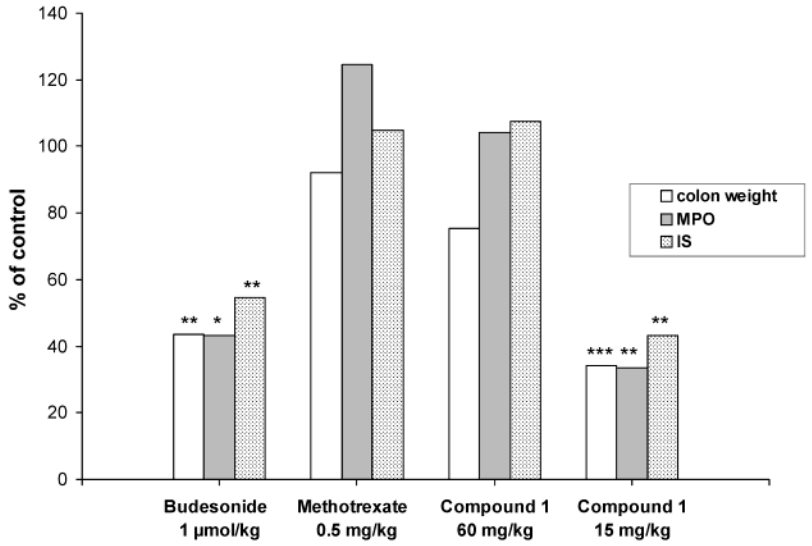


Figure 5. Effects of compound **1**, MTX, and budesonide in the DNBS mouse colitis model. Effect of treatment was assessed by three different parameters: colon weight, MPO (myeloperoxidase), and IS (inflammation scoring). The animals were treated with test compounds once daily over 7 days, and the effects were analyzed 1 day after end of treatment. The results are expressed as the percent of response in untreated control animals (mean values). Significant differences from control group are indicated: (∗) $p < 0.05$; (∗∗) $p < 0.01$; (∗∗∗) $p < 0.001$.

Purines, as well as the pyrimidines, are required for lymphocyte proliferation. Hence, inhibition of AICARtf has been proposed as one of the mechanisms for the immunosuppressive effects of MTX.

All four ester analogues (**1**−**4**) exhibit fair inhibitory activity of DHFR. Despite the modifications of the hydrophilic side chain, the 2,4-diaminoquinazolines **1**, **3**, and **4** still exhibited better inhibitory activity of recombinant hDHFR than the corresponding pteridine analogue **2**.[30] The structure of the side chain has apparently less impact on the binding of the ligand to the enzyme although derivatives **1** and **2**, comprising the L-glutamic acid residue, should more likely serve as substrates for polyglutamation than **3** and **4**.[31]

Regarding MTX and compounds **1**−**4**, the in vitro data demonstrate a discrepancy between DHFR inhibition and the ability of these compounds to inhibit cell proliferation. While MTX exerted an inhibitory effect at a minimum of 30 nM, no signs of such inhibition were found with any of the compounds **1**−**4**. With respect to the DHFR inhibition value for compound **1**, one may have expected to see inhibition of lymphocyte proliferation at approximately 8 times the dose of MTX. Unexpectedly, the only inhibitory effect on proliferation with compound **1** observed was at the highest dose tested, 30 μM. At this concentration of compound **1**, inhibition of cell proliferation was comparable to that of MTX at 30 nM. The observed discordance between DHFR binding and cell growth inhibition may reflect differences in cellular influx via the reduced folate carrier.

A significant positive effect was noted in vivo with compound **1** but surprisingly only at the lower dose of 15 mg/(kg·day). The inhibition of DHFR may play an important role for the therapeutic effect, but this is not supported by the in vivo data with compound **1** given at a 30-fold higher dose than MTX. The inverse dose response observed in vivo with compound **1** could point toward potential side effects using MTX, or analogues thereof, at the indicated doses in vivo. However, one ought to take into account that although the in vivo experiments were performed in mice, rat and mouse DHFR are highly homologous and show similar sensitivity to MTX. It is notable that a comparative pharmacokinetic study showed that the bioavailability of compound **1** after oral administration was only slighter lower than that for methotrexate, according to LC−MS.[12,30]

The colitis model used in this study is quite complex, involving several components of the inflammatory response. We have earlier also noted some experimental variation concerning the degree of inflammation from experiment to experiment. An important part of the defense to mucosal injury is the regeneration of the epithelial layer. We propose that high concentrations of metabolically stable antifolates may negatively affect the regeneration of the epithelium and thus the healing process. This could possibly explain the better effect of a lower dose, as observed with compound **1**. Negative effects would be of particular importance in experiments where the animals have a high degree of inflammation and thereby are more susceptible to interference with healing mechanisms. The data suggest that compound **1**, devoid of ability to inhibit proliferation, has beneficial antiinflammatory properties compared to MTX, although the molecular mechanisms remain to be elucidated.

Conclusion

Analogues of methotrexate in which the C9−N10 bridge has been replaced by an ester group retain much of the DHFR inhibitory activity. One of the antifolates synthesized (compound **1**) exerted a clear antiinflammatory effect in a mouse model of colitis as assessed by three different parameters. This potent ester-based antifolate analogue of methotrexate did not inhibit mouse spleen cell proliferation, implicating that the effect of this compound in vivo is less likely to be related to inhibition of lymphocyte proliferation. We conclude that the mechanism of action of this ester analogue of MTX may be different from that of MTX. The results

presented herein suggest that compounds such as **1** provide starting points for further development toward new chemical entities with lower systemic exposure and better antiinflammatory properties.

Experimental Section

Chemistry. General Information. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX270 spectrometer at 270 and 67.8 MHz, respectively, and a JEOL JNM-EX400 spectrometer at 400 and 100 MHz, respectively. Thin-layer chromatography (TLC) was performed by using aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm) type E, Merck. Chromatographic spots were visualized by UV light or an ethanolic solution of ninhydrin or an acidic ethanolic solution of *p*-anisaldehyde followed by heating. Column chromatography was conducted on silica gel S (0.032−0.063 mm, Riedel-deHaën) and silica gel 60 (0.040−0.063 mm, E. Merck) unless otherwise noted. Centrifugal chromatography was carried out on a Harrison Research Chromatotron (model 7924T) with silica gel PF_{254} containing gypsum (E. Merck) as solid phase. Melting points (uncorrected) were determined in open glass capillaries on an Electrothermal apparatus. Infrared (IR) spectra were recorded on a Perkin-Elmer 1605 FT-IR spectrophotometer and are recorded in ν_{max} (cm^{-1}). The elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden, or Analytische Laboratorien, Gummersbach, Germany, and were within ±0.4% of the calculated values. All commercial chemicals were used without further purification.

Di-*tert*-butyl L-α-Aminoadipate (5). See refs 13 and 30.

Di-*tert*-butyl *N*-(4-Formylbenzoyl)-L-glutamate (6). See refs 14 and 30.

Di-*tert*-butyl *N*-(4-Formylbenzoyl)-D-glutamate (7). See refs 15 and 30.

Di-*tert*-butyl *N*-(4-Formylbenzoyl)-L-α-aminoadipate (8). A mixture of 4-formylbenzoic acid (295 mg, 1.96 mmol), PyBOP (929 mg, 1.79 mmol), *N*,*N*-diisopropylethylamine (0.68 mL, 3.90 mmol), and CH_2Cl_2 (12 mL) was stirred together for 15 min. Di-*tert*-butyl L-α-aminoadipate **(5)** in CH_2Cl_2 (13 mL) was added, and the reaction mixture was allowed to stir overnight. Workup was performed as described for compound **6**, yielding an oil, which was purified with centrifugal chromatography [EtOAc/isohexane (1:7)] affording 665 mg (92%): ^{1}H NMR ($CDCl_3$) δ 10.06 (1H, C*H*O), 7.99−7.92 (m, 4H, Ar*H*), 7.05 (d, J = 7.26 Hz, 1H, N*H*), 4.69−4.62 (m, 1H), 2.30−2.24 (m, 2H), 2.01−1.59 (m, 4H), 1.49 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 191.53, 172.56, 171.32, 165.81, 139.16, 138.24, 129.76 (2C), 127.79 (2C), 82.60, 80.44, 53.04, 34.73, 31.59, 28.05 (3C), 27.98 (3C), 20.50. Anal. ($C_{22}H_{31}NO_6$) C, H, N.

Di-*tert*-butyl *N*-(4-Carboxybenzoyl)-L-glutamate (9). Sodium chlorite (2.29 g, 25.3 mmol) and $NaH_2PO_4 \cdot 1H_2O$ (2.71 g, 19.6 mmol) in water (30 mL) was added dropwise to a solution of **6** (1.10 g, 2.81 mmol), *tert*-butyl alcohol (50 mL), and 2-methyl-2-butene (15 mL). The reaction was finished after 3 h according to TLC. The reaction mixture was extracted between 1 M HCl and $CHCl_3$ followed by washing with 10% $Na_2S_2O_3$ and water, respectively. Subsequent drying ($MgSO_4$), filtration, concentration under reduced pressure, and purification by flash chromatography [$CHCl_3$/MeOH (19:1)] provided a white foam: yield, 1.07 g (93%); IR (CH_2Cl_2) 1725 (ester), 1649 (amide) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.35 (br s, 1H, COO*H*), 8.09−7.85 (m, 4H, Ar*H*), 7.32 (d, J = 7.59 Hz, 1H, N*H*), 4.71−4.64 (s, 1H), 2.52−2.02 (m, 4H), 1.49 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.88, 171.37, 169.97, 166.43, 138.33, 132.06, 130.30 (2C), 127.24 (2C), 82.83, 81.21, 53.18, 31.71, 28.03 (6C), 27.13. Anal. ($C_{21}N_{29}NO_7 \cdot 1.25H_2O$) C, H, N.

Di-*tert*-butyl *N*-(4-Carboxybenzoyl)-D-glutamate (10). Compound **10** was prepared from **7** (0.988 g, 2.52 mmol) as described above for compound **9**. Subsequent workup afforded a white foam: yield, 0.803 g (78%); IR (CH_2Cl_2) 1725 (ester), 1655 (amide) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.10−7.86 (m, 4H, Ar*H*), 7.31 (d, J = 7.42 Hz, 1H, N*H*), 4.71−4.64 (m, 1H), 2.53−2.02 (m, 4H), 1.50 (s, 9H), 1.42 (s, 9H). Anal. ($C_{21}H_{29}NO_7 \cdot 0.5H_2O$) C, H, N.

Di-*tert*-butyl *N*-(4-Carboxybenzoyl)-L-α-aminoadipate (11). Compound **11** was prepared from **8** (665 mg, 1.64 mmol) according to the same procedure as described for compound **9**, affording after purification by flash chromatography [$CHCl_3$/MeOH (9:1)] 643 mg (93%) of the pure carboxylic acid: IR (CH_2Cl_2) 1731 (ester), 1655 (amide) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.09−7.86 (m, 4H, Ar*H*), 7.03 (d, J = 7.42 Hz, 1H, N*H*), 4.72−4.65 (m, 1H), 2.33−2.28 (m, 2H), 2.06−1.61 (m, 4H), 1.51 (s, 9H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.74, 171.77, 169.73, 166.49, 138.16, 132.68, 130.20 (2C), 127.13 (2C), 82.73, 80.55, 53.06, 34.77, 31.57, 28.05 (3C), 27.98 (3C), 20.67. Anal. ($C_{22}H_{31}NO_7 \cdot 0.5H_2O$) C, H, N.

6-(Bromomethyl)-2,4-diaminoquinazoline Hydrobromide (12). See refs 12 and 30.

6-(Bromomethyl)-2,4-diaminopteridine Hydrobromide (13). See refs 12, 21, 22, and 30.

Di-*tert*-butyl *N*-[4-(2,4-Diaminoquinazoline-6-yl)methyl]benzoyl]-L-glutamate (14). Freshly prepared **12** (0.53 mmol) was added to a mixture of **9** (322 mg, 0.79 mmol) and K_2CO_3 (146 mg, 1.06 mmol) in dry DMAc (15 mL). The reaction mixture was stirred at room temperature for 24 h before it was filtered and evaporated to dryness on silica gel under reduced pressure. The silica plug was added on top of a silica column and the crude product was purified by repeated flash chromatography [$CHCl_3$/MeOH + $NH_{3(aq)}$ (9:1)], providing 116 mg (38% over two steps) of the pure ester: IR (KBr) 1723 (ester), 1655 (amide) cm^{-1}; ^{1}H NMR (DMF-d_6) δ 8.88 (d, J = 7.42 Hz, 1H, N*H*), 8.26 (d, J = 1.32 Hz, 1H, *H*-5), 8.16−8.09 (m, 4H, Ar*H*), 7.72 (dd, J = 8.58, 1.65 Hz, 1H, *H*-7), 7.53 (br s, 2H, NH_2), 7.33 (d, J = 8.58 Hz, 1H, *H*-8), 6.16 (br s, 2H, NH_2), 5.43 (s, 2H), 4.58−4.49 (m, 1H), 2.47 (app t, 2H), 2.26−1.99 (m, 2H), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR (DMF-d_6) δ 172.11, 171.42, 166.63, 165.76, 163.43, 161.69, 152.81, 138.90, 133.37, 132.79, 129.69 (2C), 128.22, 128.07 (2C), 124.96, 124.21, 110.55, 81.18, 80.18, 67.25, 53.40, 31.83, 27.69 (3C), 27.62 (3C), 26.63. Anal. ($C_{30}H_{37}N_5O_7 \cdot 0.75H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-(2,4-Diaminopteridine-6-yl)methyl]-benzoyl]-L-glutamate (15). Compound **15** was prepared from freshly prepared **13** (0.52 mmol) under the same conditions as described above for the synthesis of **14**. Purification by repeated flash chromatography [$CHCl_3$/MeOH + $NH_{3(aq)}$ (29:1 and 39:1)] yielded 71 mg (23% over two steps) of a light-yellow solid: IR (KBr) 1720 (ester), 1670 (amide) cm^{-1}; ^{1}H NMR (DMF-d_6) δ 9.00 (s, 1H, *H*-7), 8.88 (d, J = 7.42 Hz, 1H, N*H*), 8.21−8.01 (m, 4H, Ar*H*), 7.85 (br s, 2H, NH_2), 6.80 (br s, 2H, NH_2), 5.54 (s, 2H), 4.59−4.51 (m, 1H), 2.48 (app t, 2H), 2.22−1.95 (m, 2H), 1.46 (s, 9H), 1.43 (s, 9H); ^{13}C NMR (DMF-d_6) δ 172.11, 171.43, 166.49, 165.66, 164.28, 163.87, 156.59, 150.73, 143.31, 139.04, 132.44, 129.85 (2C), 128.15 (2C), 122.24, 81.18, 80.18, 66.13, 53.48, 31.90, 27.73 (3C), 27.68 (3C), 26.72. Anal. ($C_{28}H_{35}N_7O_7 \cdot 0.5H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-(2,4-Diaminoquinazoline-6-yl)methyl]benzoyl]-D-glutamate (16). Compound **16** was prepared from **10** (270 mg, 0.66 mmol) and as described above for compound **14**. Workup as for **14** with repeated flash chromatography [$CHCl_3$/MeOH + $NH_{3(aq)}$ (39:1 with a gradient to 29:1)] and further isolation of the product with centrifugal chromatography [CH_2Cl_2/MeOH + $NH_{3(aq)}$ (49:1)] afforded 157 mg (51% over two steps) of the desired compound: IR (KBr) 1725 (ester), 1637 (amide) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.00−7.78 (m, 4H, Ar*H*), 7.73 (d, J = 1.65 Hz, 1H, *H*-5), 7.59 (dd, J = 8.58 Hz, 1.65, 1H, *H*-7), 7.44 (d, J = 7.59 Hz, 1H, N*H*), 7.40 (d, J = 8.91 Hz, 1H, *H*-8), 6.14 (br s, 2H, NH_2), 5.33 (s, 2H), 5.15 (br s, 2H, NH_2), 4.68−4.60 (m, 1H), 2.51−2.05 (m, 4H), 1.48 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.17, 171.31, 166.30, 165.67, 162.63, 160.28, 152.18, 137.82, 133.70, 132.47, 129.66 (2C), 128.89, 127.15 (2C), 125.60, 122.62, 110.10, 82.61, 81.06, 66.85, 53.19, 31.77, 28.00 (6C), 27.05. Anal. ($C_{30}H_{37}N_5O_7 \cdot 1.5H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-(2,4-Diaminoquinazoline-6-yl)methyl]benzoyl]-L-α-aminoadipate (17). Compound **17** was prepared from **11** (333 mg, 0.79 mmol) in dry DMF (15 mL) as described above for compound **14**. Purification with flash chromatography [$CHCl_3$/MeOH + $NH_{3(aq)}$ (19:1)] was followed

by centrifugal chromatography [$CHCl_3$/MeOH + $NH_{3(aq)}$ (39:1)] to afford 106 mg (34% over two steps) of the pure ester: ^{1}H NMR ($CDCl_3$) δ 8.11−7.85 (m, 4H, Ar*H*), 7.71−7.67 (m, 2H, *H*-5 + *H*-7), 7.48 (d, J = 9.57 Hz, 1H, *H*-8), 6.98 (d, J = 7.42 Hz, 1H, N*H*), 5.57 (br s, 2H, NH_2), 5.41 (s, 2H), 4.89 (br s, 2H, NH_2), 4.70−4.63 (m, 1H), 2.28 (app t, 2H), 2.00−1.60 (m, 4H), 1.50 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.60, 171.71, 166.35, 165.63, 162.67, 160.05, 151.49, 137.96, 133.71, 132.34, 129.63 (2C), 128.94, 127.13 (2C), 125.10, 122.71, 110.01, 82.55, 80.47, 66.80, 53.10, 34.71, 31.51,28.04 (6C), 20.75. Anal. ($C_{31}H_{39}N_5O_7\cdot0.5H_2O$) C, H, N.

***N*-[4-[(2,4-Diaminoquinazoline-6-yl)methylene]oxycarbonyl]benzoyl L-Glutamic Acid (1). Method A.** Compound **14** (180 mg, 0.31 mmol) was dissolved in trifluoroacetic acid (10 mL), and the mixture was stirred at room temperature for 40 min. Evaporation under reduced pressure was followed by trituration with diethyl ether, and the precipitate was filtered. Water (4 mL) was added, and the pH was adjusted to 3.9 with 1 M NaOH. The precipitate was filtered off and washed consecutively with water, ethanol, acetone, and diethyl ether, yielding 121 mg (83%) of a white solid: IR (KBr) 1724 (ester), 1652 (amide) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.73 (d, J = 7.75 Hz, 1H, N*H*), 8.20 (app d, 1H, *H*-5), 8.10−7.98 (m, 4 + 2H, Ar*H* + NH_2), 7.83 (br s, 2H, NH_2), 7.74 (dd, J = 8.58, 1.65 Hz, 1H, *H*-7), 7.32 (d, J = 8.58 Hz, 1H, *H*-8), 7.09 (br s, 2H, NH_2), 5.37 (s, 2H), 4.42−4.34 (m, 1H), 2.35 (app t, 2H), 2.16−1.88 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 174.06, 173.84, 165.51, 165.12, 162.65, 158.54, 138.42, 133.98, 131.77, 129.28 (2C), 128.93, 127.80 (2C), 124.29, 121.27, 109.46, 66.52, 52.44, 30.72, 26.31 (one aromatic carbon missing).

Method B. Compound **14** (0.035 mg, 60 μmol) was dissolved in formic acid (5 mL) and warmed to 40 °C. TLC showed the absence of starting material after 30 min. Workup was performed as described in method A, providing 20 mg (71%). Anal. ($C_{22}H_{21}N_5O_7\cdot1H_2O$) C, H, N.

***N*-[4-[(2,4-Diaminopteridine-6-yl)methylene]oxycarbonyl]benzoyl L-Glutamic Acid (2).** Compound **2** was synthesized from compound **15** (47 mg, 81 μmol) as described above in method B, providing 30 mg (79%) of a light-yellow solid: ^{1}H NMR (DMSO-d_6) δ 8.90 (s, 1H, *H*-8), 8.79 (d, J = 7.92 Hz, 1H, N*H*), 8.12−7.98 (m, 4H, Ar*H*), 7.68 (br s, 2H, NH_2), 6.74 (br s, 2H, NH_2), 5.48 (s, 2H), 4.45−4.36 (m, 1H), 2.38−2.33 (m, 2H), 2.12−1.91 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 173.82, 173.16, 165.59, 165.05, 163.24, 162.80, 155.74, 150.33, 142.35, 138.22, 131.58, 129.35 (2C), 127.85 (2C), 121.60, 65.64, 52.11, 30.53, 25.98. Anal. ($C_{20}H_{19}N_7O_7\cdot1HCO_2H\cdot0.25H_2O$) C, H, N.

***N*-[4-[(2,4-Diaminoquinazoline-6-yl)methylene]oxycarbonyl]benzoyl D-Glutamic Acid (3).** Compound **3** was prepared from compound **16** (130 mg, 0.22 mmol) as described above in method B, affording 90 mg (86%) of a white solid: ^{1}H NMR (DMSO-d_6) δ 8.66 (d, J = 7.26 Hz, 1H, N*H*), 8.17 (s, 1H, *H*-5), 8.09−7.98 (m, 4H, Ar*H*), 7.78 (br s, 2H, NH_2), 7.70 (d, J = 8.25 Hz, 1H, *H*-7), 7.29 (d, J = 8.58 Hz, 1H, *H*-8), 6.89 (br s, 2H, NH_2), 5.37 (s, 2H), 4.41−4.34 (m, 1H), 2.37−2.31 (m, 2H), 2.14−1.88 (m, 4H). Anal. ($C_{22}H_{21}N_5O_7\cdot0.5H_2O$) C, H, N.

***N*-[4-[(2,4-Diaminoquinazoline-6-yl)methylene]oxycarbonyl]benzoyl L-α-Aminoadipic Acid (4).** Compound **4** was prepared from compound **17** (107 mg, 0.18 mmol) according to method A, yielding 66 mg (76%) of an off-white solid: ^{1}H NMR (DMSO-d_6) δ 8.81 (d, J = 7.42 Hz, 1H, N*H*), 8.58 (br s, 2H, NH_2), 8.29 (s, 1H, *H*-5), 8.11−8.00 (m, 4H, Ar*H*), 7.86 (dd, J = 8.58, 1.32 Hz, 1H, *H*-7), 7.62 (br s, 2H, NH_2), 7.43 (d, J = 8.41 Hz, 1H, *H*-8), 5.41 (s, 2H), 4.40−4.32 (m, 1H), 2.24 (app t, 2H), 1.91−1.53 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 174.26, 173.71, 165.61, 165.10, 162.85, 156.12, 138.26, 134.91, 131.73, 130.87, 129.31 (2C), 127.89 (2C), 124.41, 118.80, 109.38, 66.15, 52.66, 33.20, 30.04, 21.48 (one aromatic carbon missing). Anal. ($C_{23}H_{23}N_5O_7\cdot0.75CF_3CO_2H\cdot1H_2O$) C, H, N.

Mouse Colitis Model. Colitis was induced in C57Bl/6 mice (Möllegaards Breeding Center (Denmark) under light anesthesia. By use of a flexible polyethylene catheter (PE-4) inserted 2 cm into the distal colon via the rectum, the animals received an intracolonic instillation of 100 μL of a solution containing 4 mg of DNBS (Sigma) dissolved in a vehicle of 30% ethanol/water. This procedure was similar to what has previously been described.[32,33] For the study, animals were housed in groups according to treatments. The day after induction of the colitis, treatment was initiated, and those animals that were given treatment by rectal administration were anesthetized briefly at each dosing time. Treatments were continued for 7 days with administration of test compounds once daily. One day after the end of the treatment, the animals were euthanized by cervical dislocation. The degree of colitis was assessed by (a) analysis of tissue myeloperoxidase, (b) by the weight of the distal 4 cm of the colon, and (c) by blinded scoring, essentially as previously described.[34] The study was approved by the local ethics committee for animal experimental studies.

In Vitro Cell Proliferation Assay. Spleen cells were isolated from female Balb/C mice and stimulated with the T-cell mitogen ConA.[35] Briefly, one or two spleens were cut into small pieces and passed through a nylon mesh. Erythrocytes were lysed with ammonium sulfate, and the remaining cells were counted. The cell proliferation assay was performed in 96-well microtiter plates, and 4×10^5 cells were added to each well. One hour prior to addition of ConA (5 μg/mL), MTX and compounds **1**−**4** were added. After 48 h incubation, ^{3}H-thymidine was added, and after another 18 h, cells were harvested and the incorporation was measured in a scintillation counter. All samples were tested in triplicate, and data were obtained in two independent experiments.

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Supporting Information Available: Experimental details for the preparation of compounds **5**−**7** and **12**−**13**, pharmacokinetics of compound **1** and MTX in rat, IC_{50} values of compounds **1**−**4**, rec hDHFR. This material is available free of charge via the Internet at http://pubs.acs.org.

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