Methotrexate γ-hydroxamate derivatives as potential dual target antitumor drugs

M. Amélia Santos,[a,*] Eva A. Enyedy,[a] Elisa Nuti,[b] Armando Rossello,[b]
Natalia I. Krupenko[c] and Sergey A. Krupenko[c]

[a]*Centro de Química Estrutural, Instituto Superior Técnico, 1049-001 Lisboa, Portugal*
[b]*Università degli Studi di Pisa, Dipartimento di Scienze Farmaceutiche Via Bonanno 6, 56126 Pisa, Italy*
[c]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA*

Abstract—A series of new aminopteroyl-based hydroxamate derivatives were synthesized and tested in vitro in cell culture models as potential dual target drugs. These compounds were designed to target two families of enzymes, matrix metalloproteinases (MMP) and a folate enzyme, dihydrofolate reductase (DHFR). These enzymes are the components of two unrelated cellular pathways and they are often over-expressed in metastasizing tumors. In addition to the synthesis and full structural characterization of the hybrid molecules, we describe their inhibitory activities against a series of MMPs (MMP-2, MMP-7, MMP-9, MMP-14) and DHFR, as well as their antiproliferative activity in three cancer cell lines. The new hydroxamate derivatives of MTX proved to be effective inhibitors of MMPs and DHFR in the micromolar and nanomolar range, respectively. Furthermore, they showed strong antiproliferative activity against A549 cells (non-small cell lung carcinoma), and PPC-1 and Tsu-Pr1 prostate cancer cell lines. Therefore, based on the present results, these bi-functional drugs may be good candidates to target specific tumors in animal models due to potential combined effects on two pathways crucial for tumor development.
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1. Introduction

Zinc-containing enzymes, including carboxypeptidase A (CPA), histone deacetylases (HDACs), tumor necrosis factor α-convertase (TACE), and matrix metalloproteinases (MMPs), are attractive therapeutic targets in treatment of numerous diseases.[1] Among these enzymes, MMPs, a family of secreted or transmembrane proteases, are of particular interest due to their role in degradation of the extracellular matrix (ECM). MMPs are tightly regulated at multiple levels, including regulation of their activity by the endogenous tissue inhibitors of MMPs (TIMPs).[2,3] Under normal physiological conditions these enzymes are not highly expressed. Uncontrolled overexpression of MMPs can lead to tissue degradation and promote a variety of diseases that require tissue remodeling, including: arthritis, tumor metastasis, multiple sclerosis, and periodontal degrada-

tion.[4,5] Therefore, inhibition of these enzymes is expected to slow or prevent the progression of these diseases. A large number of synthetic MMP inhibitors (MMPIs) have been developed as potential therapeutics.[6–10] Since all the MMPs possess a catalytic zinc ion in their active site, MMP inhibitors contain a zinc-binding group (ZBG) linked to different scaffolds to assure strong interactions within the cofactor-binding region of the enzymes. The most developed class of MMPIs contains the hydroxamate as the ZBG group.[8] Several potent MMPIs have been tested for tumor treatment, but at present none of them are approved as drugs. Among the 28 MMPs characterized so far, the gelatinases (MMP-2, MMP-9), the matrilysin (MMP-7), and the Membrane-Type 1-metalloproteinase (MMP-14) are considered to play the most crucial role in promoting tumor progression, facilitating metastatic dispersion and angiogenesis.[5,6,10] Therefore, specific inhibitors of these MMPs are of high interest as potential anticancer drugs.[11]

Antifolates are another class of molecules that have been used for many years in cancer chemotherapy.[12–18]

They are the folate analogs that inhibit key folate enzymes, namely thymidylate synthase (TS) and dihydrofolate reductase (DHFR).[12] This inhibition induces cytotoxic effects by the ultimate suppression of de novo biosynthesis of purine nucleotides and thymidylate.[18] The antifolate methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid, MTX) is one of the most widely used drugs in medical oncology for treatment of several types of cancer.[19] It is also used in the treatment of inflammatory diseases such as rheumatoid arthritis.[20] MTX differs from folic acid by two substitutions: (i) a hydroxyl is replaced by an amino group at C^4 position of the pterine ring; (ii) a methyl group is added to N^{10} position (Fig. 1). Such a 'minor' difference converts the DHFR substrate into a tight-binding inhibitor.[13,21] MTX is effectively transported across the cell membrane by a carrier-mediated mechanism.[17,21,22] Upon entering the cell, it undergoes polyglutamylation that decreases its efflux and enhances inhibitory effects.[12,13,21]

Efficacy of MTX can be limited by acquired resistance that occurs through multiple mechanisms including: decreased MTX transport, diminished polyglutamylation of the drug, overexpression of its main target, DHFR, or expression of MTX-resistant DHFR mutants.[12,17,21,23] To produce more potent drugs that can circumvent resistance to MTX, novel antifolates have been developed, including a new generation of multitarget drugs.[24–28] Pemetrexed, a multitarget drug, targeting DHFR and TS, was the first antifolate approved by the US FDA (February, 2004) since the introduction of MTX more than 50 years ago.[26]

In recent years, together with development of new drugs, significant efforts have been directed toward the development of combination therapies in treatment of different types of cancers.[29–31] In this strategy, the use of drugs targeting different, often unrelated, pathways, with expectation of synergistic effects, becomes an approach of increasing interest. As a long-standing chemotherapeutic, MTX has been successfully used in combination with other drugs for quite some time.[32–34] In this regard, MMP inhibitors are not well studied although their synergistic effects with other anticancer drug have been observed in animal models.[35] Interestingly, recent phase I studies evaluated effects of an MMP inhibitor in combination with 5-fluorouracil (the TS inhibitor) and leucovorin in patients with advanced solid tumors.[36] Further development of combination therapy involves the use of multifunctional drugs, when one chemical compound can hit two or more targets.[37] Such approach has high potential to improve therapeutic efficacy. In our studies, we pursued the development of a new type of double-target drugs by combining two structurally unrelated inhibitory functions (targeting folate pathways and MMPs) in a single molecule. Since MMP-dependent tissue remodeling and folate requiring biosynthetic pathways are crucial for tumor progression, simultaneous management of both types of pathways, by a single drug, could be highly beneficial. MTX still remains an effective drug in cancer treatment, therefore in our design strategy it was selected as the antifolate compound to be modified into a series of hydroxamate derivatives. The synthesized derivatives were tested for their inhibitory activity toward DHFR and several MMPs as well as for their antiproliferative activity in cancer cell lines.

2. Molecular design

The design of the compounds, reported in the present paper, is based on the addition of extra functional moi-
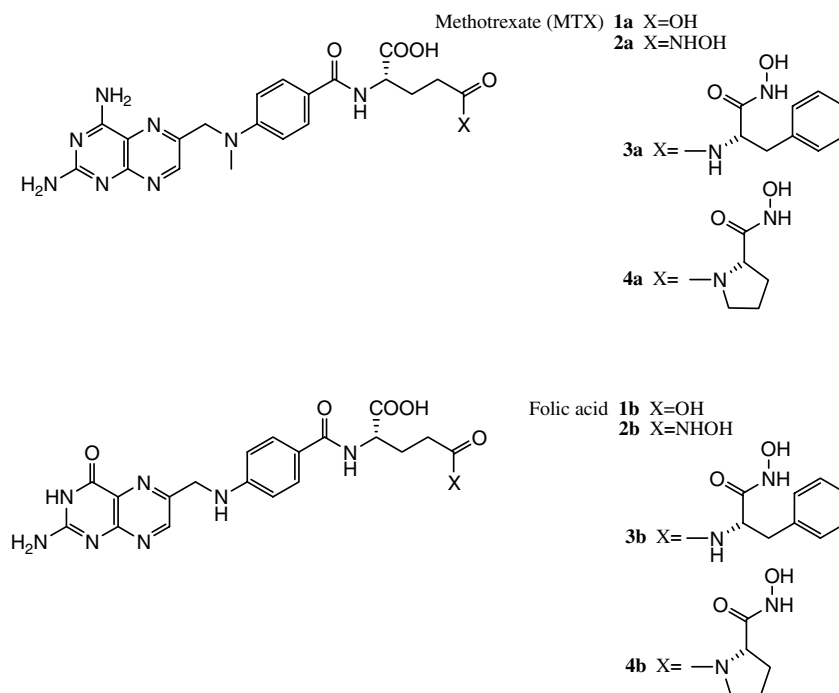

Figure 1. Structures of MTX, folic acid, and their hydroxamic acid derivatives **2a,b–4a,b**.

ety to MTX molecule. In an attempt to improve selectivity and decrease toxicity, numerous derivatives of MTX have been synthesized.[38–40] In general, only the γ-carboxyl has been modified because the free α-carboxyl group contributes significantly to the binding of MTX to DHFR. In particular, incorporation of non-polar groups such as alkylamino-acids or alkylic esters, and monohydroxamic group, at the γ-position has been performed.[40] A monohydroxamate derivative of MTX was found to be moderately toxic to human and mouse leukemia cells.[41] Thus, the main goal of the present studies was an attempt to improve MTX as anticancer drug by adding an additional function to MTX molecule.

The MTX-derivatives developed herein should be efficient inhibitors of specific MMPs in addition to their DHFR inhibition capabilities. Accordingly, the MTX structural elements, responsible for the interaction with active site of DHFR, were kept, while the hydroxamate moiety, a strong zinc-binding group (ZBG), was introduced at the γ-position of MTX glutamate (by replacement of the γ-COOH by γ-CONHOH) to enable the MMPs' inhibition. The efficacy of a MMP inhibition depends on two factors: (i) the ability to bind the catalytic zinc ion; (ii) the aptitude to form contacts with the enzyme subsites (via hydrogen and van der Waals bonds).[6,7] To enhance the capability of the inhibitors to bind to MMPs, monoamino acid spacers, containing hydrophobic residues, were introduced between the hydroxamate group and the core molecule.

Folic acid derivatives, analogous to the MTX γ-hydroxamate derivatives, were also synthesized and studied as the control compounds. Thus, the following derivatives of MTX and folic acid were synthesized and tested (see Fig. 1): methotrexate γ-L-phenylalaninehydroxamic acid (**3a**), methotrexate γ-L-prolinehydroxamic acid (**4a**), methotrexate γ-hydroxamic acid (**2a**); folate γ-L-phenylalaninehydroxamic acid (**3b**), folate γ-L-prolinehydroxamic acid (**4b**), folate γ-hydroxamic acid (**2b**).

3. Results and discussion

3.1. Chemistry

Commercial preparations of MTX and folic acid were used in our syntheses (Scheme 1). The first step was activation of the γ-carboxylic group of MTX or folic acid that was achieved by using stoichiometric amount of TBTU in the presence of *N*-methyl-morpholine. Preferential modification of the γ-carboxylic group over the α-carboxylic group was due to hydrogen bonding of the latter to the neighboring amide group and limited access of the bulky modifying agent to the α-position. Subsequent condensation with the amino acid molecules (L-Phe, L-Pro) or the free hydroxylamine (Scheme 1) yielded the corresponding MTX/folic acid peptide derivatives or the simple hydroxamate derivatives. The synthesis of the amino acid-hydroxamate derivatives involved an additional step to convert the carboxylic group of the amino acid to the corresponding hydroxa-

mic acid through condensation with free hydroxylamine. All reactions were performed in dry DMF under nitrogen atmosphere to exclude moisture. All synthesized compounds were characterized by NMR spectroscopy.

3.2. MMPs inhibition

The synthesized compounds and the reference drug CGS 27023A (used as a control) were tested as inhibitors of MMPs often overexpressed in tumors (MMP-2, MMP-7, MMP-9, and MMP-14).[6] The hydroxamic acid derivatives of folic acid and MTX showed inhibitory effects on studied MMPs in the micromolar range (Table 1). Under the same experimental conditions, GGS 27023A resulted in a much more potent inhibition with IC_{50} in the nanomolar range.

Interestingly, in the case of folic acid derivatives, the insertion of an amino acid spacer between the folate molecule and the hydroxamate group improved inhibitory potency as compared with the folate hydroxamate **2b** (Table 1). In particular, introduction of the phenylalanine spacer (**3b**) improved the inhibitory effects, especially against MMP-7 ($IC_{50} = 15$ μM) and MMP-9 ($IC_{50} = 22$ μM). Substitution of the phenylalanine by the proline spacer (**4b**) resulted in a reduction of the inhibitory effects on MMP-2 and MMP-7, although the inhibitory effects of these derivatives against MMP-9 ($IC_{50} = 30$ μM) and MMP-14 ($IC_{50} = 100$ μM) were similar. In the case of MTX hydroxamic acid derivatives, the introduction of an amino acid spacer also enhances MMP inhibitory potency. In particular, **3a** derivative shows IC_{50} values ranging from 15 μM on MMP-9 to 70 μM on MMP-14. Overall, enhanced inhibitory effects against the tested MMPs were observed when a flexible lipophilic amino acid spacer (phenylalanine-hydroxamate) was added between the core molecule and the hydroxamic group.

3.3. DHFR inhibition

The synthesized derivatives of MTX and folic acid were evaluated for the ability to inhibit activity of DHFR, the MTX target. DHFR is capable of catalyzing two reactions in folate cycle, conversion of folic acid to dihydrofolate and conversion of dihydrofolate to tetrahydrofolate.[42] The DHFR-catalyzed conversion of dihydrofolate to tetrahydrofolate is a much faster reaction than conversion of folic acid to dihydrofolate and it is viewed as a major metabolic function of DHFR. The other reaction, however, is also important if the source of folate is folic acid. In our cell culture experiments (see Experimental section) the cells were supplemented with folic acid as the only source of folate. The absorbed folic acid should be first converted to dihydrofolate in a DHFR-catalyzed reaction; accordingly, inhibition of cellular growth in our experiments is likely to be related to the inhibition of this reaction since this is the first and required step for the entry of folic acid into the reduced folate pool. Therefore, both folic acid and dihydrofolic acid were used as a substrate to evaluate DHFR inhibition in vitro. Overall, all tested MTX-based compounds produced similar inhibitory effects on both reactions.
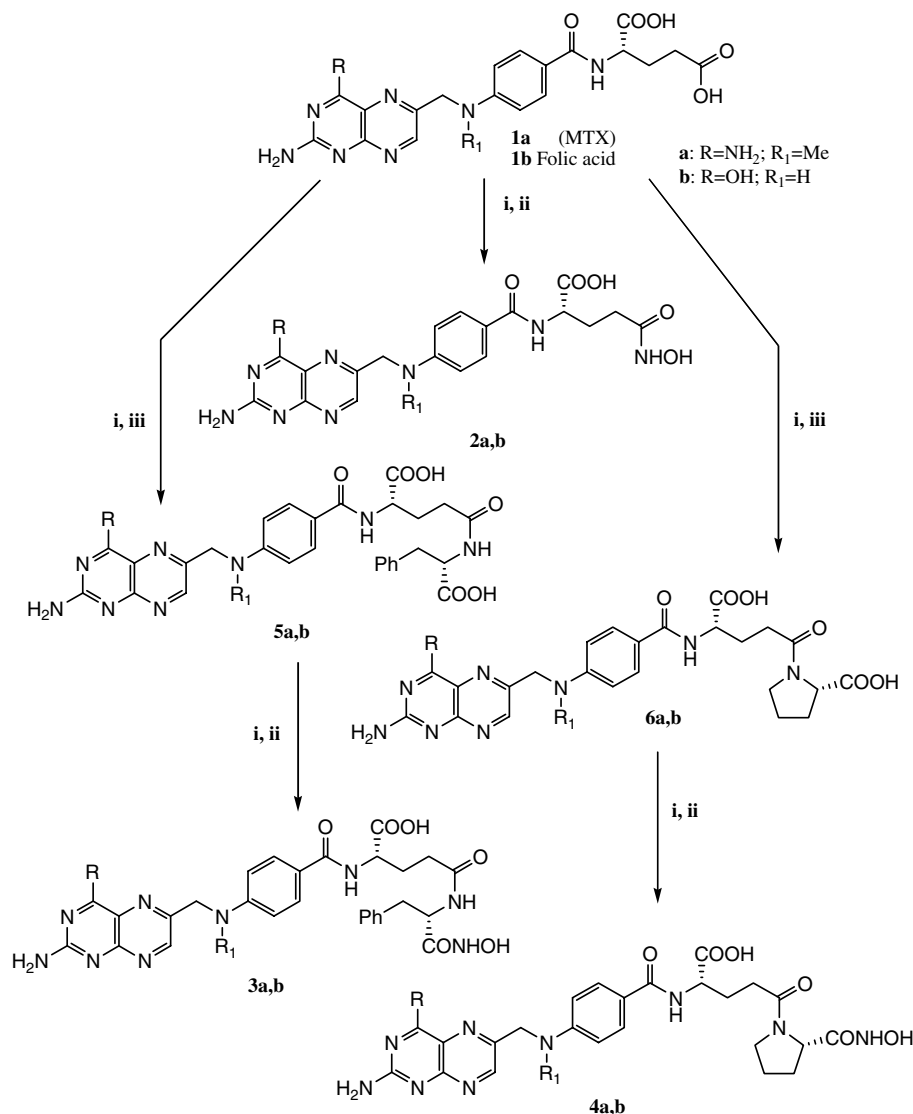
Scheme 1. Synthesis of the γ-hydroxamic acid derivatives of MTX and folic acid. Reagents and conditions: (i) TBTU, NMM, 0 °C, dry DMF; (ii) NH$_2$OH, 0 °C, dry DMF; (iii) aminoacid (L-Phe, L-Pro), 0 °C, dry DMF.

Table 1. Inhibitory activity (IC$_{50}$ μM or % inhibition at 100 μM) of a set of γ-hydroxamate derivatives of the folic acid (**2b–4b**), of the MTX (**2a–4a**), and a reference drug CGS 27023A toward the selected MMPs

Compound	MMP-2	MMP-7	MMP-9	MMP-14
2b	66%	53%	55%	42%
3b	65 (2708)[a]	15 (150)[a]	22 (4400)[a]	80 (3478)[a]
4b	58%	54%	30	100
2a	67%	100	61%	48%
3a	60 (2500)[a]	44 (440)[a]	15 (3000)[a]	70 (3043)[a]
4a	56%	43%	61%	47%
CGS 27023A	0.024	0.1	0.005	0.023

[a] IC$_{50}$-inhibitor/IC$_{50}$-CGS 27023A ratios.

These experiments revealed that the derivatives of MTX possessed strong inhibitory effects toward DHFR with IC$_{50}$ in a hundred nanomolar range (Table 2). In contrast, the folate analogs did not show notable inhibition at concentration up to 100 μM (Table 2). Compared to MTX, however, the hydroxamate derivatives of MTX displayed lower inhibitory effects on DHFR activity.

We explain this by decreasing affinity of DHFR to the derivatives, due to sterical hindrance of the substitute group in the active site of the enzyme. Indeed, **3a**, the derivative with the most bulky spacer, showed the lowest inhibitory effect. In addition, at neutral pH the carboxylate is negatively charged, while the hydroxamate group has no charge. Thus, the modification of the car-

Table 2. Antiproliferative data, IC$_{50}$ (μM), of γ-hydroxamate derivatives of folic acid and MTX on cultured cancer cells (A549, PPC-1, Tsu-Pr1); inhibitory data of MTX γ-hydroxamate derivatives, IC$_{50}$ (nM), against dihydrofolate reductase (DHFR)

Compound	Antiproliferative activity[a] IC$_{50}$ (μM)			DHFR[b] IC$_{50}$ (nM)	
	A549	PPC-1	Tsu-Pr1	Folic acid	DHF
2b	100	80	120	ND[c]	ND
3b	110	70	150	ND	ND
4b	130	120	80	ND	ND
2a	6.0	2.0	2.0	170	70
3a	6.5	4.8	2.0	250	170
4a	7.5	1.8	2.3	130	120
MTX	0.1	0.1	0.1	35	9

[a] Average of two experiments performed in triplicate is shown except for MTX-derivatives; average of three experiments performed in triplicate is shown for MTX-derivatives.
[b] DHFR was assayed using as a substrate folic acid or dihydrofolic acid (DHF).
[c] ND, non-detectable (no effects on DHFR activity were observed at up to 100 μM inhibitor concentration).

boxylic moiety of glutamic acid eliminates the negatively charged group that could contribute to binding with DHFR.

3.4. Cell proliferation assays

The antiproliferative effects of the folate and methotrexate-hydroxamate derivatives have been evaluated on three cell lines, A549 (lung carcinoma), Tsu-Pr1 and PPC-1 (prostate carcinomas). Effects of these compounds were compared to the antiproliferative effects of MTX as a control. Cell viability was assessed by MTT cell proliferation assay as described elsewhere.[43] For all three tested cell lines, we have observed that derivatives of MTX were much stronger inhibitors of proliferation than folate derivatives (Table 2). However, none of the tested inhibitors was as effective as MTX itself: IC$_{50}$ for MTX was about 0.1 μM, while IC$_{50}$ for all MTX-derivatives were in a micromolar range. Although some antiproliferative effects of the folate derivatives were observed at higher concentrations (in the range near 10^{-4} M), this could be explained by the simple competition with media folate for the folate transporters. Since concentration of folate in media (2.2 μM) is much lower than the effective concentrations of the folate derivatives, these derivatives could interfere with folate uptake. In the cell, folate derivatives apparently cannot substitute for folate in folate-required reactions, most likely due to loss of polyglutamylation.

In another set of experiments we have tested effects of MTX-derivatives on proliferation of cells grown on folate-depleted media. Regular medium is supplemented with 2.2 μM folic acid that is a high folate concentration. In experiments with lower folate supplementation, cells were grown on folate-free media supplemented with 20 nM leucovorin (5-formyltetrahydrofolate). Cells grow very slowly in low folic acid media, while leucovorin, a reduced folate, can support normal cell growth at very low concentrations.[44] At low folate supplementation, antiproliferative effects of MTX-derivatives were more profound and closer to the effects of MTX itself (Table 3). Thus, IC$_{50}$ were in the 10^{-8}–10^{-7} M range for all three cell lines. Among the three cell lines, PPC-1 cells were the most sensitive to MTX and MTX-based inhibitors, while A549 cells were the most resistant.

Table 3. IC$_{50}$ (μM) of MTX hydroxamate derivatives in cultured cancer cells (A549, PPC-1, Tsu-Pr1) grown on folate-depleted media

Compound	A549	PPC-1	Tsu-Pr1
2a	0.1	0.05	0.01
3a	0.5	0.5	0.12
4a	0.5	0.2	0.1
MTX	0.02	0.01	0.008

Average of two experiments performed in triplicate is shown.

Compound **2a**, that has the least bulky substituent, was the most effective of MTX-derivatives in inhibition of proliferation.

Overall, among the synthesized compounds, only MTX hydroxamate derivatives, but not folic acid derivatives, demonstrated strong inhibitory effects (nanomolar range) against DHFR. This suggests that the growth suppressor effects resulted from DHFR inhibition and should be attributed to MTX component. The enhancement of the suppressor effects of MTX hydroxamate derivatives in folate-depleted media further suggests that these effects are associated with the folate metabolism. More pronounced reduction of antiproliferative activity of MTX derivatives, as compared to MTX itself, than the decrease in DHFR inhibition could be an indication of diminished cellular transport of the synthesized derivatives. Alternatively, it could be due to the loss of polyglutamylation inside the cell: it is well known that polyglutamylated forms of MTX are stronger inhibitors than MTX monoglutamate.[21]

4. Conclusions

γ-Monohydroxamate derivatives of MTX, a novel type of prospective pharmacological agents with dual target capability, have been developed and tested. Our results demonstrated that the synthesized derivatives retain capability to inhibit two independent targets of metastasizing tumors, matrix metalloproteinases (MMPs) and dihydrofolate reductase (DHFR). In general, these studies demonstrated the feasibility of our approach in the development of multitarget drugs capable to inhibit two different enzyme systems. In terms of chemothera-

py, combination of two inhibitory determinants in one molecule opens an opportunity to target simultaneously proliferation of primary tumor cells and tumor invasiveness. Thus, the synthesized compounds could be good candidates for testing on experimental tumors in animal models. In general, the precautions should be taken in development of MTX-based dual target inhibitors: the preferential binding of MTX to DHFR, due to an extremely high affinity, could consume most of the intracellular inhibitor making it unavailable to target other enzymes. However, in our case compartmentalization of the selected targets allows avoiding this problem. Indeed, MMPs are extracellular enzymes, while DHFR is intracellular. Therefore, it could be expected that the synthesized compounds upon delivery to the tumors will inhibit MMPs first, preventing metastasis, and then, after translocation into tumor cells, will further inhibit proliferation by targeting DHFR.

5. Experimental

5.1. General methods

All reagents were of commercial quality and used without further purification. Folic acid, methotrexate, O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium-tetrafluoroborate (TBTU), L-phenylalanine (L-Phe), L-proline (L-Pro), N-methylmorpholine (redistilled), and hydroxylamine hydrochloride were purchased from Sigma–Aldrich. The solvents were purchased from Acros Organics or Merck; whenever necessary, they were purified and dried according to standard methods.[45] All the moisture-sensitive reactions were performed under nitrogen atmosphere. The chemical reactions were followed by TLC using silica gel plates (G-60 F_{254}, Merck). A Bio-Rad Merlin FTS 3000 MX spectrometer was used to record solid state IR spectra (KBr pellets). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 300 FT NMR spectrometer (300 MHz) at 25 °C. If necessary, assignment of the signals of the ^{13}C NMR was confirmed by DEPT. Chemical shifts are reported in ppm (δ) from sodium 3-(trimethylsilyl)-[2,2,3,3-2H_4]-propionate as internal reference in D_2O solutions. The following abbreviations are used: d, doublet; s, singlet; t, triplet; m, multiplet; br, broad. Mass spectra (FAB) were obtained on a VG TRIO-2000 GCMS instrument. High-resolution mass spectrometry (HRMS (ESI)) measurements were performed on an APEX III FT-ICR MS (Bruker Daltonics, Billerica, MA), equipped with a 7T actively shielded magnet. Ionization was achieved by an electrospray ionization source (Bruker Daltonics, Billerica, MA), with a voltage between 1800 and 2200 V (to optimize ionization efficiency) applied to the needle, and a counter voltage of 450 V applied to the capillary. Samples were prepared by adding a spray solution of 70:29.5:0.5 (v/v/v) CH_3OH/water/formic acid to a solution of the sample at a v/v ratio of 1–5% to give the best signal-to-noise ratio. Data acquisition and processing were performed using the XMASS software, version 6.1.2 (Bruker Daltonics). Elemental analyses were performed on a Fisons EA 1108 CHNF/O instrument.

5.2. Synthesis of the compounds

5.2.1. Folate γ-hydroxamic acid (2b). To a solution of folic acid (0.85 g, 2 mmol) in dry DMF (40 mL) under nitrogen was added N-methylmorpholine (0.45 mL, 4 mmol) at room temperature and then TBTU (0.65 g, 2 mmol) under cooling with an ice-water bath. This reaction mixture was stirred for 45 min at 0 °C. To a solution of hydroxylamine hydrochloride (0.42 g, 6 mmol) in dry DMF (10 mL) under nitrogen, containing activated molecular sieves (0.4 nm, Riedel-de Haën), was added KOH (0.34 g, 6 mmol). The resulting mixture was stirred for 30 min and the inorganic solids were filtered off from the solution. The first solution was slowly added to the second one and the mixture was stirred for 5 h at 0 °C and then it was kept at 0 °C for 12 h. The solvent was evaporated under vacuum, the solid residue was diluted into CH_2Cl_2 and recrystallized from dry ethanol. The resulting solid was washed with ethanol/diethyl ether to give **2b** (0.64 g, 70%) as a crystalline solid, mp 230–240 °C (decomposition). ^{1}H NMR (D_2O, pD = ca. 9) δ: 8.66 (1H, s, CH=N), 6.63 (1H, d, CH=C–NH), 7.59 (2H, d, CH=C–C=O), 4.29 (1H, s, CH–COOH), 4.47 (2H, s, CH_2–NH), 2.08 (2H, d, CH_2–CH), 2.29 (2H, t, CH_2–C=O); ^{13}C NMR (DEPT) (D_2O, pD = ca. 9) δ: 150 (CH=N), 115 (CH=C–NH), 132 (CH=C–C=O), 58.4 (CH–COOH), 48.5 (CH$_2$–NH), 36.8 (CH$_2$–CH), 31.0 (CH$_2$–C=O); IR: 1513 cm^{-1} (C=O, new amide bond); HRMS (ESI) calcd for (M+1) 457.15786; found: 457.15803.

5.2.2. Folate γ-L-phenylalanine (5b). A cooled solution containing the activated folic acid (prepared as described above) was slowly added to a water-ice cooled solution of L-phenylalanine (0.33 g, 2 mmol) in dry DMF (10 mL) and the reaction mixture was stirred for 2 h at 0 °C. After being warmed up to room temperature, the reaction mixture was evaporated in vacuo. The resulting residue was diluted with CH_2Cl_2 and recrystallized from dry ethanol to give **5b** (88% yield) as a yellow solid. ^{1}H NMR (D_2O, pD = ca. 9) δ: 8.62 (1H, s, CH=N), 6.86 (2H, d, CH=C–NH), 7.63 (2H, d, CH=C–C=O), 7.02 (5H, m, C_6H_5), 4.47 (1H, s, CH–COOH), 4.31 (1H, m, CH–CH$_2$–C_6H_5), 4.62 (2H, s, CH_2–NH), 2.01 (2H, d, CH_2–CH), 2.24 (2H, t, CH_2–C=O), 3.02 (2H, m, CH_2–C_6H_5); ^{13}C NMR (DEPT) (D_2O, pD = ca. 9), δ: 150 (CH=N), 116 (CH=C–NH), 131 (CH=C–C=O), 132 (C_6H_5), 59.0 (CH–COOH), 57.0 (CH–CH$_2$–C_6H_5), 48.3 (CH$_2$–NH), 34.1 (CH$_2$–CH), 30.1 (CH$_2$–C=O), 40.0 (CH$_2$–C_6H_5); IR: 1512 cm^{-1} (C=O, new amide bond); MS (FAB) m/z: 589 (M+1).

5.2.3. Folate γ-L-phenylalaninehydroxamic acid (3b). The title compound was prepared from **5b** according to the procedure described for **2b**. Yellow crystals (44% yield), mp 255–260 °C (decomposition). ^{1}H NMR (D_2O, pD = ca. 9) δ: 8.59 (1H, s, CH=N), 6.93 (7H, m, CH=C–NH, C_6H_5), 7.63 (2H, d, CH=C–C=O), 4.45 (1H, s, CH–COOH), 4.31 (1H, s, CH–CH$_2$–C_6H_5), 4.59 (2H, s, CH_2–NH), 1.98 (2H, d, CH_2–CH), 2.25 (2H, t, CH_2–C=O), 2.95 (2H, m, CH_2–C_6H_5); ^{13}C NMR (DEPT) (D_2O, pD = ca. 9) δ: 151 (CH=N), 116 (CH=C–NH), 131 (CH=C–C=O), 132 (C_6H_5), 58.6

(C*H*–COOH), 55.8 (CH–*C*H$_2$–C$_6$H$_5$), 48.2 (*C*H$_2$–NH), 31.9 (*C*H$_2$–CH), 30.0 (*C*H$_2$–C=O), 39.5 (*C*H$_2$-C$_6$H$_5$); IR: 1509 cm^{-1} (C=O, new amide bond); Anal. Found: C, 53.93; H, 4.94; N, 19.36%. C$_{24}$H$_{27}$N$_9$O$_7$·1.22 MeOH requires: C, 53.65; H, 4.76; N, 20.11%; HRMS (ESI) calcd for (M+1) 604.2263; found: 604.2278.

5.2.4. Folate γ-L-proline (6b). The title compound was prepared from folic acid and L-proline according to the procedure described for **5b**. Yellow crystals (81% yield); ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.60 (1H, s, C*H*=N), 6.84 (2H, d, C*H*=C–NH), 7.66 (2H, d, C*H*=C–C=O), 4.27 (1H, s, C*H*–COOH), 4.19 (1H, s, N–C*H*–CH$_2$), 4.61 (2H, s, C*H$_2$*–NH), 2.08 (6H, m, C*H$_2$*–CH, C*H$_2$*–C=O, N–CH$_2$–CH$_2$–CH$_2$), 3.56 (2H, m, N–C*H$_2$*–CH$_2$–CH$_2$), 2.47 (2H, m, N–CH$_2$–CH$_2$–C*H$_2$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 150 (*C*H=N), 115 (*C*H=C–NH), 132 (*C*H=C–C=O), 65.4 (*C*H–COOH), 50.0 (N–*C*H–CH$_2$), 47.4 (*C*H$_2$–NH), 32.7 (*C*H$_2$–CH), 29.6 (*C*H$_2$–C=O), 39.5 (N–*C*H$_2$–CH$_2$–CH$_2$), 22.6 (N–CH$_2$–*C*H$_2$–CH$_2$), 28.4 (N–CH$_2$–*C*H$_2$–CH$_2$); IR: 1513 cm^{-1} (C=O, new amide bond); MS (FAB) *m/z*: 539 (60%) (M+1).

5.2.5. Folate γ-L-proline-hydroxamic acid (4b). The title compound was prepared from **6b** according to the procedure described for **2b**. Yellow crystals (37% yield), mp 240–245 °C (decomposition). ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.59 (1H, s, C*H*=N), 6.84 (2H, d, C*H*=C–NH), 7.66 (2H, d, C*H*=C–C=O), 4.28 (1H, m, C*H*–COOH), 4.19 (1H, s, N–C*H*–CH$_2$), 4.59 (2H, s, C*H$_2$*–NH), 2.15 (6H, m, C*H$_2$*–CH, C*H$_2$*–C=O, N–CH$_2$–C*H$_2$*–CH$_2$), 3.64 (2H, m, N–C*H$_2$*–CH$_2$–CH$_2$), 2.48 (2H, m, N–CH$_2$–CH$_2$–C*H$_2$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 151 (*C*H=N), 115 (*C*H=C–NH), 131 (*C*H=C–C=O), 64.2 (*C*H–COOH), 50.2 (N–*C*H–CH$_2$), 47.9 (*C*H$_2$–NH), 34.0 (*C*H$_2$–CH), 32.1 (*C*H$_2$–C=O), 43.2 (N–*C*H$_2$–CH$_2$–CH$_2$), 24.4 (N–CH$_2$–*C*H$_2$–CH$_2$), 27.0 (N–CH$_2$–CH$_2$–*C*H$_2$); IR: 1512 cm^{-1} (C=O, new amide bond); Anal. Found: C, 50.95; H, 5.01; N, 20.94%. C$_{28}$H$_{29}$N$_9$O$_7$·0.64 HCl requires: C, 51.11; H, 5.42; N, 21.26%. HRMS (ESI) calcd for (M+1) 554.2106; found: 554.2103.

5.2.6. Methotrexate γ-hydroxamic acid (2a). The title compound was prepared from methotrexate according to the procedure described for **2b**. Recrystallization from DMF provided yellow crystals with 62% yield, mp 212–216 °C. ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.55 (1H, s, C*H*=N), 6.86 (2H, d, C*H*=C–NH), 7.69 (2H, d, C*H*=C–C=O), 4.30 (1H, s, C*H*–COOH), 4.76 (2H, C*H$_2$*–NCH$_3$), 2.08 (2H, d, C*H$_2$*–CH), 2.25 (2H, t, C*H$_2$*–C=O), 3.14 (3H, s, C*H$_3$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 152 (*C*H=N), 115 (*C*H=C–NH), 132 (*C*H=C–C=O), 58.1 (*C*H–COOH), 63.1 (*C*H$_2$–NCH$_3$), 38.7 (*C*H$_2$–CH), 31.0 (*C*H$_2$–C=O), 42.5 (*C*H$_3$); IR: 1514 cm^{-1} (C=O, new amide bond); HRMS (ESI) calcd for (M+1) 470.1895; found: 470.1907.

5.2.7. Methotrexate γ-L-phenylalanine (5a). The title compound was prepared from methotrexate and L-phenylalanine according to the procedure described for **5b**. Yellow crystals (75% yield); ^{1}H NMR (D$_2$O,

pD = ca. 9) δ: 8.53 (1H, s, C*H*=N), 6.84 (2H, d, C*H*=C–NH), 7.67 (2H, d, C*H*=C–C=O), 7.29 (5H, C$_6$*H$_5$*), 4.26 (1H, s, C*H*–COOH), 4.18 (1H, s, C*H*–CH$_2$–C$_6$H$_5$), 4.74 (2H, s, C*H$_2$*–NCH$_3$), 2.04 (2H, d, C*H$_2$*–CH), 2.26 (2H, t, C*H$_2$*–C=O), 2.87 (2H, m, C*H$_2$*–C$_6$H$_5$), 3.13 (3H, s, C*H$_3$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 152 (*C*H=N), 115 (*C*H=C–NH), 130 (*C*H=C–C=O), 131 (*C*$_6$H$_5$), 58.3 (*C*H–COOH), 57.3 (*C*H–CH$_2$–C$_6$H$_5$), 68.6 (*C*H$_2$–NCH$_3$), 34.7 (*C*H$_2$–CH), 29.5 (*C*H$_2$–C=O), 41.5 (*C*H$_2$–C$_6$H$_5$), 44.9 (*C*H$_3$); IR: 1511 cm^{-1} (C=O, new amide bond); MS (FAB) *m/z*: 602 (~100%) (M+1).

5.2.8. Methotrexate γ-L-phenylalaninehydroxamic acid (3a). The title compound was prepared from **5a** according to the procedure described for **2b**. Yellow crystals (83% yield), mp 190–195 °C. ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.58 (1H, s, C*H*=N), 7.65 d (2H, d, C*H*=C–C=O), 6.92 (7H, m, C$_6$*H$_5$*, C*H*=C–NH), 4.41 (1H, s, C*H*–COOH), 4.27 (1H, s, C*H*–CH$_2$–C$_6$H$_5$), 4.67 (2H, s, C*H$_2$*–NCH$_3$), 1.96 (2H, d, C*H$_2$*–CH), 2.17 (2H, t, C*H$_2$*–C=O), 2.82 (2H, m, C*H$_2$*–C$_6$H$_5$), 3.22 (3H, s, C*H$_3$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 152 (*C*H=N), 115 (*C*H=C–NH), 131 (*C*H=C–C=O), 132 (*C*$_6$H$_5$), 57.2 (*C*H–COOH), 56.4 (*C*H–CH$_2$–C$_6$H$_5$), 68.7 (*C*H$_2$–NCH$_3$), 35.6 (*C*H$_2$–CH), 29.8 (*C*H$_2$–C=O), 39.0 (*C*H$_2$–C$_6$H$_5$), 43.1 (*C*H$_3$); IR: 1510 cm^{-1} (C=O, new amide bond); HRMS (ESI) calcd for (M+1) 617.2579; found: 617.2582.

5.2.9. Methotrexate γ-L-proline (6a). The title compound was prepared from methotrexate and L-proline according to the procedure described for **5b**. Yellow crystals (58% yield); ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.56 (1H, s, C*H*=N), 6.86 (2H, d, C*H*=C–NH), 7.67 (2H, d, C*H*=C–C=O), 4.40 (1H, s, C*H*–COOH), 4.23 (1H, s, N–C*H*–CH$_2$), 4.59 (2H, s, C*H$_2$*–NCH$_3$), 2.05 (6H, m, C*H$_2$*–CH, C*H$_2$*–C=O, N–CH$_2$–C*H$_2$*–CH$_2$), 3.51 (2H, m, N–C*H$_2$*–CH$_2$–CH$_2$), 2.45 (2H, m, N–CH$_2$–CH$_2$–C*H$_2$*), 3.15 (3H, s, C*H$_3$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 152 (*C*H=N), 114 (*C*H=C–NH), 131 (*C*H=C–C=O), 57.4 (*C*H–COOH), 50.3 (N–*C*H–CH$_2$), 64.4 (*C*H$_2$–NCH$_3$), 33.5 (*C*H$_2$–CH), 32.0 (*C*H$_2$–C=O), 41.3 (N–*C*H$_2$–CH$_2$–CH$_2$), 26.6 (N–CH$_2$–*C*H$_2$–CH$_2$), 29.6 (N–CH$_2$–CH$_2$–*C*H$_2$), 42.8 (*C*H$_3$); IR: 1510 cm^{-1} (C=O, new amide bond); MS (FAB) *m/z*: 552 (~100%) (M+1).

5.2.10. Methotrexate γ-L-proline-hydroxamic acid (4a). The title compound was prepared from **6a** according to the procedure described for **2b**. Yellow crystals (56% yield), mp 233–235 °C. ^{1}H NMR (D$_2$O, pD = ca. 9) δ: 8.58 (1H, s, C*H*=N), 6.90 (2H, d, C*H*=C–NH), 7.66 (2H, d, C*H*=C–C=O), 4.40 (1H, m, C*H*–COOH), 4.21 (1H, s, N–C*H*–CH$_2$), 4.61 (2H, s, C*H$_2$*–NCH$_3$), 1.99 (6H, m, C*H$_2$*–CH, C*H$_2$*–C=O, N–CH$_2$–C*H$_2$*– CH$_2$), 3.48 (2H, m, N–C*H$_2$*–CH$_2$–CH$_2$), 2.49 (2H, m, N–CH$_2$–CH$_2$–C*H$_2$*), 3.16 (3H, s, C*H$_3$*); ^{13}C NMR (DEPT) (D$_2$O, pD = ca. 9) δ: 152 (*C*H=N), 115 (*C*H=C–NH), 131 (*C*H=C–C=O), 57.5 (*C*H–COOH), 49.3 (N–*C*H–CH$_2$), 68.4 (*C*H$_2$–NCH$_3$), 35.2 (*C*H$_2$–CH), 31.1 (*C*H$_2$–C=O), 38.7 (N–*C*H$_2$–CH$_2$–CH$_2$), 25.7 (N–CH$_2$–*C*H$_2$–CH$_2$), 27.9 (N–CH$_2$–CH$_2$–*C*H$_2$), 42.2 (*C*H$_3$); IR:

1509 cm^{-1} (C=O, new amide bond); HRMS (ESI) calcd for (M+1) 567.2423; found: 567.2437.

5.3. Cell culture studies

The lung carcinoma cell line A549 was obtained from American Type Culture Collection. The prostate carcinoma cell lines Tsu-Pr1 and PPC-1 were a kind gift from Dr. James Norris, Medical University of South Carolina. Cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate (complete medium). In experiments with depleted folate supplementation, cells were grown on folate-free media supplemented with 10% dialyzed PBS and 20 nM leucovorin. All cells were grown at 37 °C under humidified air containing 5% CO$_2$. Cells were plated in 96-well plates at a density of about 10,000 cells/well. Treatment with different concentrations of a corresponding inhibitor was performed constantly for 72 h. MTT cell proliferation assay was performed using CellTiter 96 kit (Promega) according to manufacturer's directions.

5.3.1. Assays of DHFR activity.
DHFR activity, in the presence and in the absence of inhibitors, has been assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm due to oxidation of NADPH to NADP$^+$. The enzyme, purified from MTX-resistant L. casei strain, was a kind gift from Dr. Priest. L. casei DHFR has a fold similar to the human enzyme, possesses the conserved residues important for MTX binding, and it has been used in a number of studies as a surrogate of human DHFR demonstrating a similar inhibitory pattern for MTX and its analogs.[46–50] All assays were performed at 30 °C in a Shimadzu 2401PC double-beam spectrophotometer. The reaction mixture contained 100 μM of folic acid or 25 μM of dihydrofolic acid, 150 μM NADPH, and 1.0–2.5 μg/mL (0.006–0.015 U/ml) DHFR. The reaction was started by the addition of the enzyme in a final volume of 1.0 ml and read against a blank cuvette containing all components except the enzyme. MTX-derivatives were added in the reaction mixture to final concentration 0.001–1.0 μM. Derivatives of folic acid were tested in the concentration range of 0.1–100 μM.

5.4. MMP inhibition assays[51]

Recombinant human progelatinases A (pro-MMP-2) and B (pro-MMP-9) were supplied by Prof Gillian Murphy (Department of Oncology, University of Cambridge, UK). Matrilysin (pro-MMP-7, human recombinant) and Membrane-Type1-MMP (pro-MMP-14, human recombinant) were purchased from Calbiochem. Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37 °C for pro-MMP-2, APMA 1 mM for 1 h at 37 °C for pro-MMP-7 and pro-MMP-9) and with trypsin 5 μg/mL for 15 min at 37 °C followed by soybean trypsin inhibitor (SBTI) 23 μg/ml for pro-MMP-14. For the assay, the stock solutions (100 mM) of the inhibitors in DMSO were further diluted, at seven different concentrations (1 nM–300 μM) for each MMP in the fluorimetric assay buffer (FAB): Tris 50 mM, pH 7.5, NaCl 150 mM, CaCl$_2$ 10 mM, Brij 35 0.05%, and DMSO 1%. The activated enzyme (final concentration 2.8 nM for MMP-2, 2.36 nM for MMP-7, 2.7 nM for MMP-9, and 2.15 nM for MMP-14) and the inhibitor solutions were incubated in the assay buffer for 4 h at 25 °C. After addition of 200 μM solution of the fluorogenic substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ (Sigma), in DMSO (final concentration 2 μM), the hydrolysis was monitored every 15 s, for 20 min, recording the increase in fluorescence (λ_{ex} = 328 nm, λ_{em} = 398 nm) using a Molecular Device M-2 Gemini plate reader. The assays were performed in duplicate in a total volume of 200 μl per well in 96-well microtiter plates (Corning, black, NBS). Control wells lack an inhibitor. The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor. IC$_{50}$ was determined using the formula: $V_i/V_o = 1/(1 + [I]/IC_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and V_o is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software and GraFit software.[52,53]

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