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Development of methotrexate proline prodrug to overcome resistance by MDA-MB-231 cells

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ABSTRACT

The resistance to methotrexate by a number of cancer cells such as breast cancer cell-line MDA-MB-231 due to poor permeability renders it less effective as an anticancer agent for these cells. Proline prodrug of methotrexate (Pro-MTX) was designed as a substrate of prolidase which is specific for imido bond of dipeptide containing proline and expected to penetrate MDA-MB-231 cells more efficiently. The prodrug was synthesized by solid-phase peptide synthesis method and examined as a substrate of pure prolidase as well as cell homogenate. The cytotoxicity against MDA-MB-231 and non-methotrexate resistant breast cancer cell line, MCF-7 was also examined by XTT assay. The results showed that Pro-MTX was a substrate of prolidase. It was also shown that the prodrug could be converted to parent drug methotrexate in Caco-2 and HeLa cell homogenate. When tested with Caco-2 and MCF-7 cells, Pro-MTX showed weaker cytotoxicity compared with methotrexate. But for methotrexate resistant MDA-MB-231 cells, Pro-MTX showed stronger activity than methotrexate. The results indicated that the proline prodrug of methotrexate may overcome the resistance of human breast cancer cells in culture.

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For chemotherapeutic drugs, poor selectivity between neoplastic cells and normal cells, often leading to adverse toxicity and narrower therapeutic window, has been a huge problem for many years. In addition, drug resistance occurs due to multiple mechanisms ranging from diminished cellular uptake to increased efflux to inactivation of active agents in cancerous cells.[1–3] Prodrugs can be designed to target cancer cells over-expressing enzymes which are able to release the parent drugs to achieve enhanced therapeutic selectivity and efficacy.[4,5] Drugs with poor permeability can be transformed to highly permeable drugs by making them into prodrugs that are substrates of transporters to improve cellular uptake. Many studies have proved the feasibility and efficiency of this approach.[6,7]

Methotrexate (MTX) is an antitumor agent being used extensively in cancer chemotherapy since 1948. The mechanism of action is well understood at molecular level. The drug is transported into cells by the reduced folate transporter and polyglutamylated to prevent efflux, and exerts its cytotoxic effect by inhibiting dihydrofolate reductase (DHFR). Adverse effects associated with methotrexate include severe damage to normal cells and organs, narrow therapeutic index, poor selectivity for neoplastic cells, multidrug resistance due to down-regulation of methotrexate polyglutamation, and decreased reduced folate carrier (RFC) mediated uptake.[8–10] Methotrexate resistance by certain cancer cells significantly restricts the effectiveness of the drug, and this phenomenon has drawn attention from many researchers. Breast cancer cell-line MDA-MB-231 is one of the cell lines resistant to methotrexate due to low expression level of reduced folate carrier (RFC) which can uptake methotrexate into the cells. Methotrexate coupled with cell penetrating peptide has been developed to overcome MTX resistance in MDA-MB-231 cells.[10] It is also found that proline prodrug of melphalan can be better transported into MDA-MB-231 cells compared with the parent drug-melphalan. Melphalan is released from its prodrug, Melphalan-proline, in MDA-MB-231 cells by a specific cytosolic imidodipeptidase, prolidase [E.C.3.4.13.9] and responsible for the cytotoxicity against MDA-MB-231.[11,12] Although prolidase needs a dipeptide substrate with small molecular weight, it is found that prolidase may have broader substrate specificity than thought previously.[13] For example, *N*-(anthraquinone-2-carbonyl)-L-proline, a relatively larger molecule, could evoke susceptibility to the action of prolidase comparable to glycyl-L-proline (GLY-PRO, standard substrate for prolidase).[13] It is possible that the proline prodrug of methotrexate could also be transported into MDA-MB-231 and released by the similar mechanisms and overcome the methotrexate resistance due to the deficiency of RFC. The proline prodrug of methotrexate, Proline–Methotrexate (Pro–MTX), is synthesized by solid-phase chemistry with high purity.

The solid-phase synthesis of the L-proline prodrug of methotrexate, Pro-MTX (**1**) was carried out in a stepwise fashion on Wang resin as described in Scheme 1.[14] Purity of the prodrug was determined by HPLC. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weight of the

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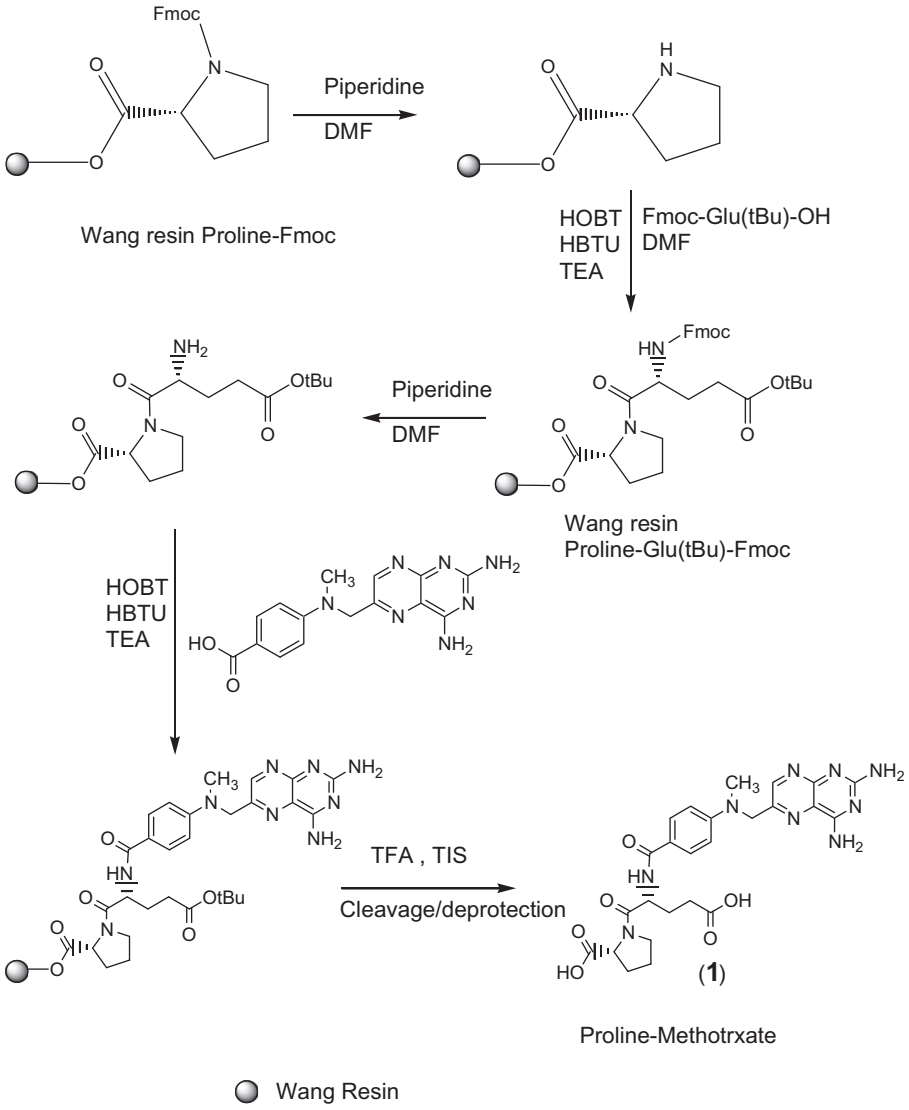

Scheme 1. Synthesis of Pro-MTX (**1**).

prodrug was found to be identical to that required by the structure. Structural identity was also confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR). ^{1}H NMR spectra were obtained with a 400 MHz Bruker NMR spectrometer.[15]

Methotrexate and prodrug were analyzed by reversed phase chromatography performed at room temperature. Detection wavelength was 274 nm. Samples were injected onto a C18 column (Xorbax, Eclipse XDB-C18, 4.6 × 75 mm, 3.5 μm, Agilent, CA, Part No. WAT066224) equipped with a analytical guard cartridge system (Phenomenex, cartridge 4.0 × 3.0, CA) and eluted at a flow rate of 0.6 ml/min. Mobile phase consisted of 0.1% TFA in water/methanol:water (50:50) (85/15). The HPLC equipment consisted of a Waters 717 plus autosampler, Waters 2695 separation module, Waters 2998 (Photodiode Array Detector), and Empower 2 software (Water Corporation) data handling system.

The commercially available prolidase from porcine kidney is similar to human prolidase (97% identity) so it is used in hydrolysis studies to observe the conversion from prodrug to parent drug. Prolidase solution was prepared by suspending the lyophilized solid (0.8 mg solid, 0.551 mg protein) in 50 mM cold Tris–HCl buffer (pH 8.0 at 40 °C) to yield a 5 mg/ml solution. The assays and enzyme activation were carried out according to protocol provided by the manufacturer (Sigma/Aldrich, St. Louis, MO). First, two sets of reagents (A and B) were prepared. Reagent A has 50 mM Tris–HCl buffer, 200 mM manganese chloride, 30 mM glutathione, and porcine kidney prolidase solution. Reagent A was incubated for 30 min at 40 °C to activate the enzyme. Reagent B was prepared by mixing 1 mM Pro-MTX and 200 mM manganese chloride. After activation, reagent A was added to reagent B at 40 °C. Reagent A without prolidase was mixed with reagent B to serve as control. Cbz-Pro was also added in Reagent B at the same concentration of Pro-MTX as inhibitor. Samples were withdrawn after 15 min, 30 min, 1 h and added into same volume of 10% TFA to quench the reaction. Then the samples were analyzed by colorimetric method.

The hydrolysis of Pro-MTX to methotrexate was also determined in Caco-2 and HeLa cell lines to check the prolidase activity in these cells. The cells were grown as described in cell culture preparation. Cell homogenate were prepared when the cells were 90% confluent.[16] Hydrolysis studies were conducted with 500 μl supernatant at 37 °C. To activate the prolidase, 500 μl of Reagent A with 50 mM Tris–HCl buffer, 200 mM manganese chloride, 30 mM glutathione were added and incubated for 2 h at 37 °C. 1 mM of Pro-MTX was added and 200 μl of samples were withdrawn after 15, 30, 60 min and added into same volume of 10% TFA to quench the reaction. The samples were then centrifuged

at 5000 rpm for 5 min at 4 °C. Two hundred microliters of the supernatant were analyzed by colorimetric method. In competitive inhibition study, Cbz-Pro with the same concentration as Pro-MTX was added as inhibitor.

By measuring the amount of proline released from the hydrolysis of Pro-MTX, we can determine the extent of the hydrolysis and calculate the hydrolysis rate. Chinard's reagent (25 g of ninhydrin in 600 ml of glacial acetic acid and 400 ml of 6 M *o*-phosphoric acid) was used according to Myara et al.[17] 200 μl of the test sample was added into 200 μl of Chinard's reagent and 200 μl of glacial acetic acid. The mixture was incubated at 90 °C for 10 min and 200 μl of the mixture was then withdrawn for UV absorbance measurement at 495 nm.

The specific activity of Pro-MTX for porcine kidney prolidase calculated from assays of released proline was 19.03 ± 1.31 pmol/min/μg. In the presence of the prolidase inhibitor Cbz-Pro, the specific activity decreased to 8.15 ± 1.10 pmol/min/μg with inhibition of 57%. The specific activity of Pro-MTX in various cancer cell homogenate was calculated from assays of released proline. Caco-2 and HeLa cells exhibited similar prolidase activity (0.42 and 0.35 pmol/min/μg protein, respectively). The hydrolysis of Pro-MTX in the presence of Cbz-Pro was significantly inhibited in all cancer cell homogenates examined. In Caco-2 cells, the specific activity was reduced to 0.28 pmol/min/μg protein with inhibition of 33%. In HeLa cells, the specific activity was reduced to 0.19 pmol/min/μg protein with inhibition of 46% (Table 1).

Methotrexate prodrug, Pro-MTX was examined for their cytotoxicity against Caco-2, MCF-7, or MDA-MB-231 cells to compare with the parent drug, methotrexate.[18] Cell viability was plotted as a function of drug concentration in the following figures. When tested with Caco-2 cells, Pro-MTX shows weaker activity compared with methotrexate. Pro-MTX has estimated EC_{50} value of 5.6 μM while methotrexate has estimated EC_{50} value of 1.1 μM. This is also confirmed by other studies showing methotrexate amino acid prodrugs are generally weaker than the parent drug for their anti-proliferation activity. The results for MCF-7 cells are similar with Caco-2 cells. Methotrexate has estimated EC_{50} value of 0.9 μM while Pro-MTX shows estimated EC_{50} value of 5.2 μM. But for methotrexate resistant MDA-MB-231 cells, Pro-MTX shows stronger activity than methotrexate. Pro-MTX has estimated EC_{50} value of 11 μM while methotrexate shows estimated EC_{50} value of 80 μM. The results indicate that the proline prodrug of methotrexate may overcome the resistance of human breast cancer cells in culture (Figs. 1–3).

Prodrug strategy has been used to target specific enzymes that are highly expressed in certain tissues to reduce toxicity and increase efficacy of the drug. Prolidase is one of the enzymes being studied extensively. It was found by Amidon et al. that prolidase activities with GLY-PRO as substrate in different cancerous cells correlate very well with the expression levels of prolidase in these cells.[5] Proline prodrugs of melphalan and others with or without linker were synthesized and studied by Bielawska[11–13,19–21] and Amidon[5,22,23] et al. The prodrugs were found to be good substrate of prolidase with significant cytotoxicity against MCF-7, Melanoma, and MDA-MB-231 cell lines. It is found that the proline prodrug of melphalan was more effectively transported into the MDA-MB-231 cells with higher cytotoxicity compared to melphalan.[12] The nature of the transport is unknown currently, but proline should play an important role in improving transport. Other proline prodrugs, such as proline-linked nitrosoureas, also exhibit

Table 1
Specific activity of Pro-MTX in the presence and absence of Cbz-Pro in prolidase and various cancer cell lines (expressed as pmol/min/μg of protein, mean ± SD, $n = 3$)

Cell lines	Specific activity with out Cbz-Pro	Specific activity with Cbz-Pro	% Inhibition
Prolidase	19.03 ± 1.31	8.15 ± 1.10	57
Caco-2	0.42 ± 0.11	0.28 ± 0.08	33
HeLa	0.35 ± 0.08	0.19 ± 0.07	46

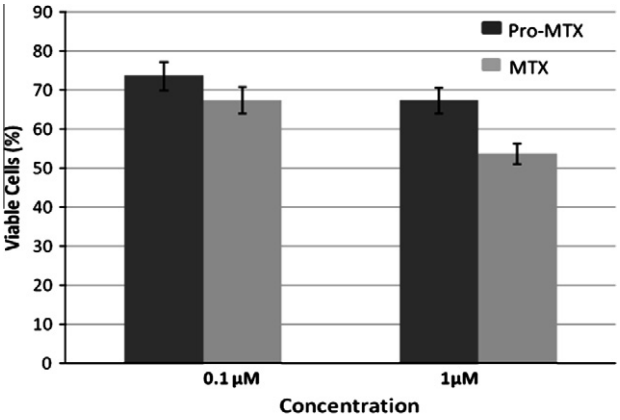

Figure 1. Cytotoxic study of methotrexate and Pro-MTX in Caco-2 cells viability of Caco-2 cells treated for 24 h with different concentrations of methotrexate and Pro-MTX (mean ± SD, $n = 3$).

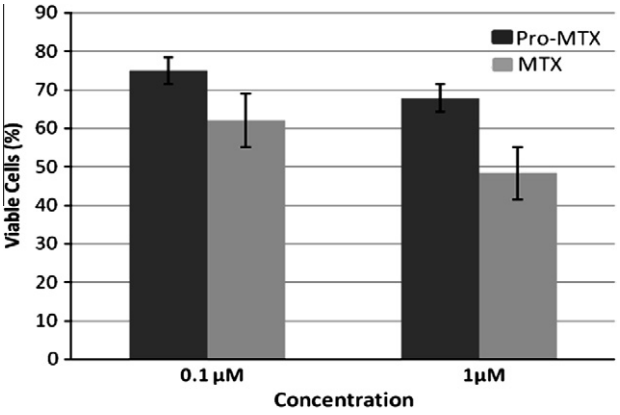

Figure 2. Cytotoxic study of methotrexate and Pro-MTX in MCF-7 cells viability of MCF-7 cells treated for 24 h with different concentrations of methotrexate and Pro-MTX (mean ± SD, $n = 3$).

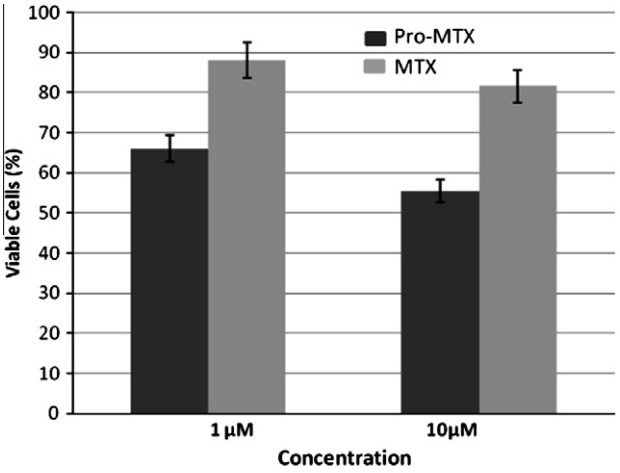

Figure 3. Cytotoxic study of methotrexate and Pro-MTX in MDA-MB-231 cells viability of MDA-MB-231 cells treated for 24 h with different concentrations of methotrexate and Pro-MTX (mean ± SD, $n = 3$)..

stronger cytotoxicity against MDA-MB-231 cell line compared to MCF-7. Whether the proline improved cellular transport is not confirmed, but it is found that the prolidase activity in MDA-MB-231 cell line was threefold higher than in MCF-7 cell.[20] MDA-MB-231 was obtained from a patient who developed resistance while being treated with methotrexate. It is an ideal cell line to study methotrexate resistant cancer.[24] The mechanism of the resistance was found to be lack of reduced folate carrier (RFC) expressed on MDA-MB-231 and that makes it more attractive to overcome the resistance by a prodrug utilizing other transport route.

In this study, we have synthesized the proline prodrug of methotrexate using solid-phase peptide synthesis method. To specifically synthesize the prodrug coupling the amino group of proline and α-carboxylic acid group of methotrexate, we utilized the γ-protected glutamate. The acid-labile *t*-butyl protecting group of glutamate was easily removed when the prodrug is cleaved off from Wang resin in acidic condition. The α-coupling is preferred over γ-coupling to mimic natural dipeptide substrate. It is also proposed that γ-carboxylic acid contribute more to the binding with DHFR than α-carboxylic acid. This would increase the possibility for Pro-MTX to exhibit cytotoxicity without being converted to methotrexate itself.

Pro-MTX showed good chemical stability in pH 7.4 PBS buffer without the presence of prolidase or other hydrolyzing enzymes (data not shown). In hydrolysis studies with prolidase, Pro-MTX was found to be a modest substrate. Caco-2 and HeLa cell homogenates were also capable of hydrolyzing Pro-MTX to release proline. This suggests that enzymes contribute significantly to the hydrolysis of the Pro-MTX. In addition to prolidase, other enzymes may also play important roles in hydrolysis of Pro-MTX. For example, carboxypeptidase A was found to be able to activate a number of amino acid methotrexate prodrugs effectively.[25] Other possible enzymes include prolylcarboxypeptidase and carboxypeptidase P.[26] Although prolidase is thought to require a dipeptide substrate with small molecular weight,[27] it is found that prolidase may have a broader substrate specificity than thought previously.[13,21]

Proline analog of methotrexate evokes weaker cytotoxicity than methotrexate in Caco-2 and MCF-7 Cells. This is consistent with other researcher's results.[10] The intrinsic cytotoxicity of methotrexate prodrugs was lower compared to that of the parent drug even we purposely synthesized the α-coupling prodrug to improve the chance to bind to DHFR. Resistance to chemotherapy limits the effectiveness of anticancer drugs. For MDA-MB-231 cell line, which is resistant to methotrexate due to compromised RFC, Pro-MTX improved cytotoxicity significantly compared to methotrexate itself. This suggests proline prodrug of methotrexate may overcome the resistance of MDA-MB-231 cell. The increased cytotoxicity might be due to two mechanisms. First, Pro-MTX could be more effectively transported into the MDA-MB-231 cells. Although the exact mechanism is not known, transporters may play important roles here. It has been proven that many amino acid coupled nucleosides including valcyclovir and valganciclovir could be transported into cells as substrates of peptide or nucleoside transporters.[28,29] Lindgren et al. discovered that methotrexate can be successfully delivered by the new cell penetrating peptide into MDA-MB-231 cell line to improve cytotoxicity.[10] Secondly, it is also possible that higher prolidase activity in MDA-MB-231 cell line (threefold higher than that in non-methotrexate-resistant MCF-7 cell) contributes to release of the more cytotoxic methotrexate in the cells. The combination of both mechanisms is also possible to be responsible for the observed results. Further studies such as uptake study using cell lines over-expressing peptide or nucleoside transporters could be carried out to elucidate the exact mechanisms.

In this study, we designed and synthesized proline prodrug of methotrexate (Pro-MTX) to target methotrexate resistant MDA-MB-231 which has higher prolidase activity and proline prodrug permeability. The results indicated that the proline prodrug of methotrexate may overcome the resistance of human breast cancer cells in culture.

References and notes

1. Stavrovskaya, A. A. *Biochemistry* **2000**, *65*, 95.
2. Nielsen, D.; Maare, C.; Skovsgaard, T. *Gen. Pharmacol.* **1996**, *27*, 251.
3. Lowenthal, R. M.; Eaton, K. *Hematol. Oncol. Clin. North Am.* **1996**, *10*, 967.
4. Huang, P. S.; Oliff, A. *Curr. Opin. Genet. Dev.* **2001**, *11*, 104.
5. Mittal, S.; Song, X.; Vig, B. S.; Landowski, C. P.; Kim, I.; Hilfinger, J. M.; Amidon, G. L. *Mol. Pharm.* **2005**, *2*, 37.
6. Lee, V. H. L. *Eur. J. Pharm. Sci.* **2000**, *11*, S41.
7. Sadee, W.; Drubbisch, V.; Amidon, G. L. *Pharm. Res.* **1995**, *12*, 1823.
8. Siotnak, F.; Burchall, J.; Ensminger, W.; Montgomery, J. *Folate Antagonists Ther. Agents* **1984**, *2*, 166.
9. Siotnak, F.; Burchall, J.; Ensminger, W.; Montgomery, J. *Folate Antagonists Ther. Agents* **1984**, *2*, 133.
10. Lindgren, M.; Rosenthal-Aizman, K.; Saar, K.; Eiríksdóttir, E.; Jiang, Y.; Sassian, M.; Östlund, P.; Hällbrink, M.; Langel, Ü. *Biochem. Pharmacol.* **2006**, *71*, 416.
11. Chrzanowski, K.; Palka, J. *Folia Histochem. Cytobiol.* **2001**, *39*, 209.
12. Chrzanowski, K.; Bielawska, A.; Palka, J. *Farmaco* **2003**, *58*, 1113.
13. Chrzanowski, K.; Bielawska, A.; Palka, J. *Rocz. Akad. Med. Bialymst.* **1998**, *43*, 201.
14. Fmoc–proline–Wang resin (200 mg, 0.1 mmol) was added in a reaction tube with filter and washed three times with 5 ml of 20% piperidine in DMF solution for 10 min to remove protecting group Fmoc. Then it was washed with DMF solution three times. HOBT (40.53 mg, 0.3 mmol), HBTU (113.79 mg, 0.3 mmol), Fmoc-Glu(OtBu)-OH (127.5 mg, 0.3 mmol) and triethylamine (TEA) (30.57 mg, 0.3 mmol) in DMF solution were added to react for 1 h on shaker. After draining the solution, the resin was washed with DMF three times and 20% piperidine in DMF three times, respectively, to remove Fmoc. HOBT (40.53 mg, 0.3 mmol) and HBTU (113.79 mg, 0.3 mmol), 4-[*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methyl amino] benzoic acid hemi hydrochloride hydrate (127.5 mg, 0.3 mmol) and triethylamine (TEA) (30.57 mg, 0.3 mmol) in DMF solution were added to react for overnight on shaker. After draining the solution, the resin was washed with DMF three times and methanol three times, respectively, and dried in vacuum. After the resin was completely dry, 5 ml of mixture (95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane) was added to react for 90 min on shaker. The solution was filtered into a 15 ml falcon tube and blew with nitrogen gas until the volume was reduced to ~200 μl. Ten milliliters of cold diethylether was added to obtain yellow precipitation. The suspension was centrifuged for 10 min at 2000 rpm and the supernatant was removed to obtain dry powder as Pro-MTX.
15. Spectroscopic data and yield of Pro-MTX:
Pro-MTX (**1**): yield, 45%; percent purity, 95%; ^{1}H NMR (DMSO): 1.70–1.95 (m, 4H, CH_2CH_2COOH, proline CH_2CH_2), 2.11 (m, 2H, proline CH_2CH_2), 3.13 (m, 2H, CH_2CH_2COOH), 3.57 (m, 2H, proline N–CH_2), 3.27 (s, 3H, N–CH_3), 4.15 (m, 1H, α-H), 4.34 (m, 1H, α-H), 4.76 (s, 2H, CH_2-N–CH_3), 6.70 (d, 2H, $CH_{benzine}$–C–N), 7.65 (d, 2H, $CH_{benzine}$–C–CO), 8.61 (s, 1H, pteroyl ring); ESI-MS: 552 $(M+H)^+$.
16. All cell lines were cultured at 37 °C with 5% CO_2 and 90% relative humidity. MCF-7 and MDA-MB-231 cells were cultured in 90% minimum essential medium (MEM) with 10% FBS. Caco-2 cells were cultured in 80% minimum essential medium (MEM) with 20% FBS. HeLa cells were cultured in 90% DMEM with 10% FBS. Split confluent culture 1:4 to 1:6 every 3–5 days using trypsin/EDTA. After trypsinization, the cells were washed three times with pH 7.4 PBS buffer and re-suspended in pH 7.4 PBS (10 mM). To prepare cell homogenate, add 1% Triton-X 100 in PBS solution and vortex vigorously. The cell suspension was centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant was used in hydrolysis study and to determine protein content. Total protein was quantified with the BioRad Protein Assay using bovine serum albumin as standard. The protein content was adjusted to ~1000 μg/ml by appropriate dilutions before being used in hydrolysis studies.
17. Myara, I.; Charpentier, C.; Lemonnier, A. *Clin. Chim. Acta* **1982**, *125*, 193.
18. For cytotoxicity study, 500 μl of Caco-2, MCF-7, or MDA-MB-231 cell in medium was cultured in 24 well plates. Generally, cells should be seeded at a density of 5×10^4 cells per well. It was allowed to grow for 48 h at 37 °C and 5% CO_2. Culture media was replaced by fresh media in each well. The synthesized prodrugs and methotrexate with different concentrations were added and incubated for 24 h. Hundred microliters of XTT mixture was added in each well and incubate for 4 h. Growth medium alone was used as controls. UV absorbance of the samples was measured at 490 nm.
19. Chrzanowski, K.; Bielawska, A.; Bielawski, K.; Woczynski, S.; Palka, J. *Farmaco* **2001**, *56*, 701.
20. Bielawski, K.; Bielawska, A.; Słodownik, T.; Bołkun-Skórnicka, U.; Muszyńska, A. *Pharmacol. Rep.* **2008**, *60*, 171.
21. Bielawska, A.; Bielawski, K.; Pałka, J. *Rocz. Akad. Med. Bialymst.* **1997**, *42*, 148.
22. Mittal, S.; Tsume, Y.; Landowski, C. P.; Lee, K. D.; Hilfinger, J. M.; Amidon, G. L. *Eur. J. Pharm. Biopharm.* **2007**, *67*, 752.
23. Mittal, S.; Song, X.; Vig, B. S.; Amidon, G. L. *Pharm. Res.* **2007**, *24*, 1290.
24. Worm, J.; Kirkin, A. F.; Dzhandzhugazyan, K. N.; Guldberg, P. *J. Biol. Chem.* **2001**, *276*, 39990.
25. Kuefner, U.; Lohrmann, U.; Montejano, Y. D.; Vitols, K. S.; Huennekens, F. M. *Biochemistry* **1989**, *28*, 2288.

26. Walter, R.; Simmons, W. H.; Yoshimoto, T. *Mol. Cell. Biochem.* **1980**, *18*, 111.
27. Bai, J. P.; Hu, M.; Subramanian, P.; Mosberg, H. I.; Amidon, G. L. *J. Pharm. Sci.* **1992**, *81*, 113.
28. Beutner, K. R. *Antiviral Res.* **1995**, *28*, 281.
29. Cocohoba, J. M.; McNicholl, I. R. *Ann. Pharmacother.* **2002**, *36*, 1075.