New Mechanisms for an Old Drug; DHFR- and non-DHFR-mediated Effects of Methotrexate in Cancer Cells

Nové možnosti starého léku: DHFR- a non-DHFR-mediované účinky metotrexátu na nádorové buňky

Neradil J.[1,2], Pavlasova G.[1], Veselska R.[1,3]

[1] Laboratory of Tumor Biology, Department of Experimental Biology, School of Science, Masaryk University, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Department of Pediatric Oncology, University Hospital Brno and School of Medicine, Masaryk University, Brno, Czech Republic

Summary
Methotrexate, a structural analogue of folic acid, is one of the most frequently used chemotherapeutics, especially in haematological malignancies, various solid tumours and also inflammatory disorders. Methotrexate interferes with folate metabolism, mainly by inhibition of dihydrofolate reductase, resulting in the suppression of purine and pyrimidine precursor synthesis. The depletion of nucleic acid precursors seems to be responsible for the cytostatic, cytotoxic and differentiation effects of methotrexate. Methylation of biomolecules represents another folate-dependent pathway that is also affected by methotrexate. Furthermore, methotrexate is able to modify metabolic pathways and cellular processes independently of folate metabolism. Based on the similar structure of methotrexate and of functional groups of certain histone deacetylase inhibitors, the ability of methotrexate to inhibit histone deacetylases was predicted and consequently verified. Recently published findings also suggest that methotrexate affects glyoxalase and antioxidant systems. Although methotrexate has been used as a folate metabolism antagonist in anticancer therapy for more than 60 years, the identification of its' other molecular targets in cellular metabolism still continues.

Key words
methotrexate – folate metabolism – dihydrofolate reductase – methylation – histone deacetylase inhibitors – glyoxalase system – oxidative stress

Souhrn
Metotrexát, strukturální analog kyseliny listové, je jedním z nejčastěji používaných chemoterapeutik především pro léčbu hematoonkologických onemocnění, solidních nádorů, ale také některých autoimunitních poruch. Primárně metotrexát narušuje folátový metabolizmus inhibicí dihydrofolátreduktázy, což má za následek potlačení syntézy pyrimidinových a purinových prekurzorů. Nedostatek stavebních kamenů nukleových kyselin se pak odráží v cytostatickém, cytotoxickém a diferenciačním efektu metotrexátu. Mezi další procesy, které jsou ovlivněny inhibicí folátového metabolizmu, patří metylace biomolekul, především proteinů a DNA. Metotrexát však působí na metabolické dráhy a buněčné procesy i nezávisle na metabolizmu folátů. Na základě podobnosti struktury metotrexátu a funkčních skupin některých inhibitorů histondeacetyláz bylo predikováno a poté i experimentálně potvrzeno, že metotrexát má schopnost inhibovat histondeacetylázy. Dále byla prokázána schopnost metotrexátu účinně ovlivňovat glyoxalázový a antioxidační systém. I když je metotrexát používán jako folátový antagonista v protinádorové terapii více než 60 let, odhalování jeho dalších cílů působení na molekulární i buněčné úrovni stále pokračuje.

Klíčová slova
metotrexát – folátový metabolizmus – dihydrofolátreduktáza – metylace – inhibitory histondeacetylázy – glyoxalázový systém – oxidativní stres

This study was supported by European Regional Development Fund and the State Budget of the Czech Republic for RECAMO (CZ.1.05./2.1.00/03.0101) and by internal project of Masaryk University No. MUNI/C/0803/2011.

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101) a interním projektem Masarykovy univerzity MUNI/C/0803/2011.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Jakub Neradil, RNDr., Ph.D.
Laboratory of Tumor Biology
Institute of Experimental Biology
School of Science
Masaryk University
Kotlarska 2
611 37 Brno
Czech Republic
e-mail: jneradil@sci.muni.cz

Submitted/Obdrženo: 1. 10. 2012
Accepted/Přijato: 6. 11. 2012

Introduction

Methotrexate (MTX; amethopterin; 4-amino-10-methylfolic acid), a structural analogue of folic acid, is one of the most frequent chemotherapeutic drugs [1]. MTX is used in the treatment of haematological malignancies, various types of solid tumours and also of inflammatory disorders. This large group of MTX-treated diseases includes leukaemia, breast cancer, colorectal cancer, head and neck cancer, lymphoma, osteogenic sarcoma, urothelial cancer, choriocarcinoma, psoriasis and rheumatoid arthritis [2]. This review is focused on the various mechanisms of MTX action at the cellular level.

Folate Metabolism

The main biochemical function of folate, especially of its reduced form tetrahydrofolate (THF), is to serve as a co-factor/ /co-enzyme and to transfer one-carbon groups. THF acts as a donor of these groups in several interconnected metabolic pathways in the cytoplasm (Fig. 1). Three of one-carbon substituted THF derivatives are associated with crucial metabolic pathways: 5-methyl THF, which is required for synthesis of methionine; 5,10-methylene THF, which is essential for the synthesis of deoxythymidylate (dTMP), a pyrimidine component of DNA; and 10-formyl THF, which serves as co-factor for purine synthesis [3,4].

MTX as Inhibitor of Nucleotide Biosynthesis

The enzyme dihydrofolate reductase (DHFR) is the key intracellular target of MTX in folate metabolism. DHFR catalyses the reduction of folate to THF in two steps. The inhibition of DHFR by MTX is competitive with dihydrofolate (DHF) and results in THF depletion, leading to the inhibition of purine and pyrimidine precursor synthesis [5].

The lack of 5,10-methylene THF is a cause of the reduced synthesis of pyrimidine precursors, because thymidylate synthase (TS) is not able to catalyse methylation of dUMP to dTMP without 5,10-methylene THF. Moreover, TS is directly blocked by MTX and by un-metabolised dihydrofolate [6]. A severe lack of dTTP can lead to the phenomenon called "thymineless stress" followed by "thymineless death" due to the inhibition of DNA synthesis. Preceding thymineless death, a large increase in dUTP concentration and its incorporation into DNA instead of dTTP can be found. Activation of the DNA excision repair pathway is the next step; however, this process cannot run correctly and apoptosis is induced by DNA damage [7]. Alternatively, changes in the ratio of intracellular concentrations of the nucleotides (i.e. nucleotide pool imbalance) are also able to trigger the mitochondrial pathway of apoptosis [8]. Nevertheless, other studies show that numerous homologous recombinations resulting from single--strand breaks in DNA are responsible for the cell death [9].

Purine precursor biosynthesis is also partially indirectly inhibited by deficiency of another folate co-factor, 10-formyl THF. However, it is primarily inhibited directly by the excessive levels of DHF in a cell [10], because during the inhibition of DHFR, the intracellular concentration of 10-formyl THF is maintained up to 80% [11]. In addition, MTX is also a direct inhibitor of AICAR transformylase (ATIC) [12] and GAR transformylase (GART) [13], two pivotal enzymes responsible for purine precursor synthesis. Unlike DHFR, the inhibition of ATIC and GART is markedly improved by polyglutamylation of MTX as MTX polyglutamates are more potent inhibitors – polyglutamy-

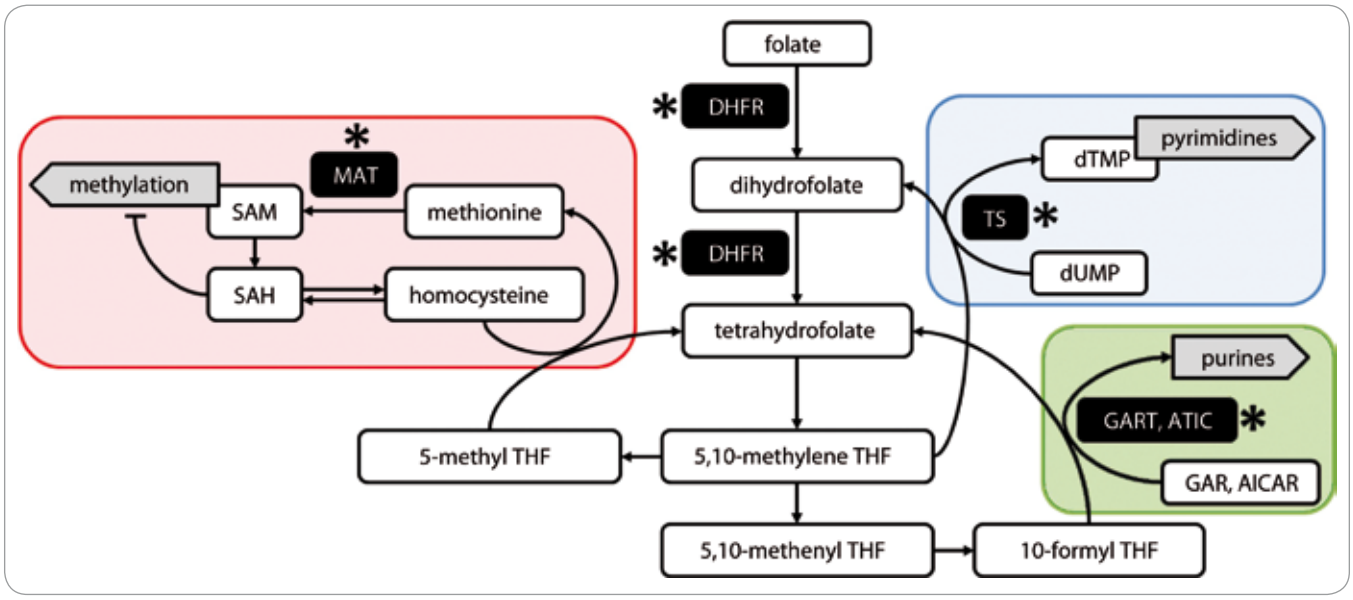

Fig. 1. Folate metabolism. Schematic picture of three main folate-dependent pathways: methylation of biomolecules (red area), thymidylate synthesis (blue area) and synthesis of purines (green area). The spots of MTX-intervention are indicated by asterisks. Abbreviations: AICAR – 5-aminoimidazole-4-carboxamide ribonucleoside; ATIC – AICAR transformylase; DHFR – dihydrofolate reductase; GAR – glycinamide ribonucleotide; GART – GAR transformylase; MAT – methionine S-adenosyltransferase; SAM – S-adenosyl methionine; SAH – S-adenosyl homocysteine; THF – tetrahydrofolate; TS – thymidylate synthase.

lated MTX provides a stronger bond with enzymes [12,14].

MTX as Inducer of Cell Death

The previous data show that the inhibition of dTMP synthesis and *de novo* purine synthesis, either directly or as a result of the inhibition of DHFR, is the main reason for MTX-induced cell death. The proportion of the inhibition effect of purine or pyrimidine precursor synthesis on cell death may differ among various cell types, as well as between two main ways of cell death – apoptosis and necrosis [15]. Apoptosis is probably initiated during the S-phase of the cell cycle when DNA is synthesised [6], because a blockade of transition from G1 to S phase prevents MTX-induced apoptosis [16].

Surprisingly, MTX can also induce apoptosis in post-mitotic cells, in which DNA replication does not occur. For example, this phenomenon was described in post-mitotic pulmonary artery endothelial cells [17], or in rodent cortical neurons [18]. Kruman et al [18] found that MTX induces cell cycle re-entry in neurons; it was confirmed by the incorporation of BrdU (5-bromo-2'-deoxyuridine) into newly synthesised DNA. Subsequently, affected cells can undergo apoptosis. The same effect was shown by homocysteine (Hcy), which additionally increased expression of p53 and cdc25 required for a progression from G1 to the S phase.

MTX as Inducer of Differentiation

Besides the cytostatic and cytotoxic effects of MTX, there was also described a differentiation effect of this compound. MTX was found to be a potent differentiation inducer in HL-60 human promyelocytic leukaemia cells [19], LA-N-1 human neuroblastoma cells [20], human neonatal foreskin keratinocytes [21], U937 human monocytic cells [22], human and rat choriocarcinoma cells [23,24], HT29 colon cancer cells [25], A549 adenocarcinoma cells [26], human APL (acute promyelocytic leukaemia) and ALL (acute lymphoblastic leukaemia) cell lines, and patients' ALL blasts [27].

The cause of the induced differentiation is not still fully understood. In some cases, differentiating effects of MTX result from thymine nucleotide depletion, because the addition of thymidine is able to prevent MTX-induced differentiation [28]. On the contrary, cell differentiation arises apparently due to the deprivation of purines in HT29 human colon cancer cells [25].

In both cases, the differentiating effect of MTX is linked to the nucleotide precursor synthesis arrest. This phenomenon was also observed in mouse [29] and human [30] embryonic stem cells, when they were intravitreally transplanted to induce neuronal differentiation in murine retinas. Furthermore, intravitreally or intraperitoneally administered MTX decreased proliferative activity and tumourigenic potential of transplanted embryonic stem cells and it also induced neuronal differentiation.

MTX as Inhibitor of Methylation of Biomolecules

One of the important folate metabolites is 5-methyl THF, which is – together with homocysteine – necessary for the endogenous synthesis of methionine. Methionine reacts with ATP and S-adenosyl methionine (SAM) is formed. SAM functions as donor of methyl groups for protein methylation (including histones), cytosine bases in DNA (CpG islands), neurotransmitters, phospholipids and other small molecules [31]. MTX decreases the level of 5-methyl THF in a cell via the functional suppression of DHFR [32,33]. Moreover, MTX directly inhibits the expression and activity of the methionine S-adenosyltransferase (MAT), which is a key enzyme catalysing the synthesis of SAM from methionine [34].

At the molecular level, Ras protein was identified to be a subject of MTX-induced hypomethylation [35]. Ras hypomethylation results in the mis-localisation of this protein from the plasma membrane to the cytoplasm, as well as a decrease of activation of ERK and AKT kinases that play a significant role in cell proliferation and differentiation. However, the inhibition of Ras methylation by MTX is not direct. It is caused by the suppression of isoprenylcysteine carboxyl methyltransferase, which is the enzyme blocked by S-adenosyl homocysteine (SAH). SAH arises in a reversible reaction from homocysteine, which cannot be methylated to methionine due to the inhibition of folate metabolism.

MTX also acts as a demethylating agent in highly methylated cutaneous T-cell lymphoma (CTCL) lines and in circulating tumour cells from a patient with leukemic CTCL [36]. In these cells, MTX reduced the methylation of CpG islands in the Fas promoter leading to its higher expression and increased sensitivity to Fas-mediated apoptosis.

Generally, the reduction of DNA methylation after the treatment with MTX usually occurs in intensively rapidly proliferating cells, such as during physiological processes of embryonic development, haematopoiesis and tissue regeneration, but also in transformed cells. In case of an insufficient pool of methyl donors, hemimethylated spots arise in DNA after mitotic division and after the next cycle there are no methyl templates on both strands of DNA of daughter cells. This process can lead to the loss of DNA methylation patterns and consequently to changes in gene expression [37].

Based on the findings mentioned above, MTX is considered to be a methylation inhibitor that could be used in the treatment of cancers with a specific DNA methylation pattern. Hypermethylated CpG sites in genes (and/or their promoters) regulating tissue development, differentiation and tumourigenesis were described in rhabdomyosarcoma [38], medulloblastoma [39], glioma [40,41] and other human cancers [42].

MTX as Inhibitor of Histone Deacetylases (HDAC)

Due to the similar structure of MTX and of functional groups of certain HDAC inhibitors (HDACi), it was predicted that MTX may have the ability to inhibit HDAC [43]. Some known HDACi, such as trichostatin (TSA) and suberoylanilide hydroxamic acid (SAHA) contain a hydrophobic group (benzyl) in their molecule. This group is connected by a short spacer (aliphatic group) with a functional group (hydroxamic acid) that acts as a chelator of Zn ion in the active site of zinc-dependent HDAC [44,45]. In con-

trast to TSA and SAHA, butyrate, the smallest HDACi, consists of 3-carbon chain linked to a carboxyl group.

MTX contains a pteridine ring, which is the hydrophobic group. Additionally, the residue of p-aminobenzoic acid is structurally similar to the TSA and SAHA. Furthermore, the end of the MTX molecule contains the residue of butyrate. It was demonstrated by computer modelling that MTX is able to bind into the binding site of HDAC homolog (HDAC-like protein) and to interact with the zinc ion and the surrounding structures of this protein. The inhibition of HDAC was also shown under *in vitro* conditions in cell lines derived from lung cancer, cervical or stomach cancer; an increase in the acetylation status of histone H3 was also described in these cell lines [43].

In addition to the acetylation of histone H3, MTX has the ability to induce the acetylation of p53 protein at residues Lys373/382 [46]. However, this posttranslational modification was not observed if other HDACi were applied. Simultaneously with the acetylation, MTX induced the phosphorylation of p53 protein at Ser15 that leads to the accumulation and increasing stability of p53 protein because acetylated sites are used in the process of its ubiquitination. HDAC-inhibiting activity of MTX resulted in down-regulation of the histone-lysine N-methyltransferase (EZH2), which is the catalytic core protein in the Polycomb Repressor Complex 2 (PRC2). PRC2 catalyses the addition of three methyl groups to Lys27 of histone 3 and mediates gene silencing of the tumour suppressor genes [47]. The epigenetic suppression of EZH2 expression by MTX resulted in the increasing expression of E-cadherin, which participates in the reduced cell migration and restricts a neoplastic transformation of epithelial cells [46].

Although the application of HDACi is a promising strategy to counter epigenetic changes associated with tumourigenesis [48,49], combination of these compounds with MTX has a different effect depending on the inhibitor type. For example, SAHA and sodium butyrate (NaBu) seem to be suitable HDACi for combination with MTX in ALL cell lines. These inhibitors increase both the cytotoxicity of MTX and the induction of apoptosis by modulation of the expression of enzymes involved in folate metabolism. After treatment with NaBu or SAHA, DHFR and TS expression decreased and the expression of the folylpoly-γ-glutamate synthetase (FPGS) was enhanced [50]. FPGS is the key enzyme which links glutamate residues to MTX and prevents MTX exclusion from the cell and increases its efficiency [4].

Nevertheless, the main problem of combined treatment with HDACi and MTX seems to be the sequence of their administration because the effects can be opposite [51,52]. Some HDACi (e.g. valproate or MS275) can even enhance the resistance of cells to MTX by up-regulation of thymidylate synthase expression; it was demonstrated in mouse choroid plexus carcinoma cell lines [53].

MTX as Inhibitor of the Glyoxalase System

Recently, it was also found that MTX affects the glyoxalase system. This three-step metabolic pathway is localised in the cytoplasm and it is considered to be the main pathway of methylglyoxal detoxification. Methylglyoxal, a secondary product of glycolysis or lipid peroxidation, is converted to D-lactate via the intermediate S-d-lactoylglutathione. The glyoxalase system consists of two enzymes, glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2) and a catalytic amount of reduced glutathione [54].

Enhanced activity or expression of Glo1 was described as a marker of many human neoplasias. This metabolic change is associated with increased invasiveness, metastatic potential and multidrug resistance [54]. Moreover, amplification of the gene encoding Glo1 was identified in some types of primary solid tumours [55].

Bartyik et al [1] showed that MTX inhibits Glo1 *in vitro*; confirmed indirectly by detection of decrease in plasma D-lactate following MTX treatment in ALL patients. Inhibition of Glo1 elevates the intracellular methylglyoxal level that causes glycation of biomolecules [56,57], production of ROS, or genotoxic damage in tumour cells [58,59]. All these changes can lead to the enhancement of antitumor effects of MTX.

Thus, the glyoxalase system, namely the Glo1 enzyme, represents another target of the anti-neoplastic actions of MTX and expands the range of MTX effects on various metabolic pathways.

MTX as Inductor of Oxidative Stress

Several studies have confirmed the role of oxidative stress in the cytotoxic effect of MTX [60–62]. It was demonstrated that some NAD(P)H-dependent dehydrogenases, namely 2-oxoglutarate, iso-citrate, malate and pyruvate dehydrogenases, are inhibited by MTX [63]. Inhibition of these enzymes can induce a decrease in the NADPH levels; NADPH is required to reduce oxidised glutathione (GSSG) to the reduced form (GSH). GSH acts as cytoplasmic antioxidant and its MTX-induced decrease leads to a reduced effectiveness of the antioxidant defence system [64]. At the tissue level, a decline of GSH, superoxide dismutase and catalase activities were observed after MTX application in rat cerebellum [65].

Association of MTX-induced apoptosis and MTX-induced ROS generation was depicted in HL-60 and Jurkat T human leukaemia cells [2]. Cell death was mediated by the mitochondrial pathway accompanied with a disruption of the mitochondrial membrane potential and subsequent activation of caspases. Another study showed that MTX activates JNK kinase through production of ROS resulting in induction of pro-apoptotic target genes and increased sensitivity to apoptosis [66].

Conclusion

Recent promising strategies in cancer treatment are based on the administration of drugs in combination and with different modes of action (cytostatics, differentiation inducers and angiogenic growth factors) [67] or on the new compounds affecting multiple, sometimes unrelated, cancer cell targets [68], because drugs designed exclusively against individual molecular targets usually cannot combat complex diseases such as cancer [69].

Although MTX has been used as a folate metabolism antagonist in cancer

therapy for more than 60 years, identification of the whole spectrum of its' molecular targets in cellular metabolism still continues. MTX inhibits not only synthesis of nucleotides and methylation of biomolecules, but also negatively regulates acetylation of histones, glyoxalase metabolism and antioxidant systems. Interventions in all of these metabolic pathways can induce changes in gene expression and consequently can lead to differentiation or cell death of cancer cells.

References

1. Bartyik K, Turi S, Orosz F et al. Methotrexate inhibits the glyoxalase system in vivo in children with acute lymphoid leukaemia. Eur J Cancer 2004; 40(15): 2287–2292.
2. Huang CC, Hsu PC, Hung YC et al. Ornithine decarboxylase prevents methotrexate-induced apoptosis by reducing intracellular reactive oxygen species production. Apoptosis 2005; 10(4): 895–907.
3. Lamprecht SA, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nat Rev Cancer 2003; 3(8): 601–614.
4. Assaraf YG. Molecular basis of antifolate resistance. Cancer Metastasis Rev 2007; 26(1): 153–181.
5. Fotoohi AK, Albertioni F. Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells. Leuk Lymphoma 2008; 49(3): 410–426.
6. Genestier L, Paillot R, Quemeneur L et al. Mechanisms of action of methotrexate. Immunopharmacology 2000; 47(2–3): 247–257.
7. Webley SD, Welsh SJ, Jackman AL et al. The ability to accumulate deoxyuridine triphosphate and cellular response to thymidylate synthase (TS) inhibition. Br J Cancer 2001; 85(3): 446–452.
8. Muñoz-Pinedo C, Robledo G, López-Rivas A. Thymidylate synthase inhibition triggers glucose-dependent apoptosis in p53-negative leukemic cells. FEBS Lett 2004; 570(1–3): 205–210.
9. Waldman BC, Wang Y, Kilaru K et al. Induction of intrachromosomal homologous recombination in human cells by raltitrexed, an inhibitor of thymidylate synthase. DNA Repair 2008; 7(10): 1624–1635.
10. Allegra CJ, Hoang K, Yeh GC et al. Evidence for direct inhibition of de novo purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. J Biol Chem 1987; 262(28): 13520–13526.
11. Allegra CJ, Fine RL, Drake JC et al. The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. Evidence for direct inhibition of purine synthesis. J Biol Chem 1986; 261(14): 6478–6485.
12. Allegra CJ, Drake JC, Jolivet J et al. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci U S A 1985; 82(15): 4881–4885.
13. Baggott JE, Morgan SL, Vaughn WH. Differences in methotrexate and 7-hydroxymethotrexate inhibition of folate-dependent enzymes of purine nucleotide biosynthesis. Biochem J 1994; 300(3): 627–629.
14. Allegra CJ, Chabner BA, Drake JC et al. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 1985; 260(17): 9720–9726.
15. McGuire JJ. Anticancer antifolates: current status and future directions. Curr Pharm Des 2003; 9(31): 2593–2613.
16. Genestier L, Paillot R, Fournel S et al. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. J Clin Invest 1998; 102(2): 322–328.
17. Merkle CJ, Moore IM, Penton BS et al. Methotrexate causes apoptosis in postmitotic endothelial cells. Biol Res Nurs 2000; 2(1): 5–14.
18. Kruman II, Wersto RP, Cardozo-Pelaez F et al. Cell cycle activation linked to neuronal cell death initiated by DNA damage. Neuron 2004; 41(4): 549–561.
19. Bodner AJ, Ting RC, Gallo RC. Induction of differentiation of human promyelocytic leukemia cells (HL-60) by nucleosides and methotrexate. J Natl Cancer Inst 1981; 67(5): 1025–1030.
20. Ross SA, Jones CS, De Luca LM. Retinoic acid and methotrexate specifically increase PHA-E-lectin binding to a 67-kDa glycoprotein in LA-N-1 human neuroblastoma cells. Int J Cancer 1995; 62(3): 303–308.
21. Schwartz PM, Barnett SK, Atillasoy ES et al. Methotrexate induces differentiation of human keratinocytes. Proc Natl Acad Sci U S A 1992; 89(2): 594–598.
22. Seitz M, Zwicker M, Loetscher P. Effects of methotrexate on differentiation of monocytes and production of cytokine inhibitors by monocytes. Arthritis Rheum 1998; 41(11): 2032–2038.
23. Hatse S, Naesens L, De Clercq E et al. Potent differentiation-inducing properties of the antiretroviral agent 9-(2-phosphonylmethoxyethyl) adenine (PMEA) in the rat choriocarcinoma (RCHO) tumor cell model. Biochem Pharmacol 1998; 56(7): 851–859.
24. Hohn HP, Linke M, Denker HW. Adhesion of trophoblast to uterine epithelium as related to the state of trophoblast differentiation: in vitro studies using cell lines. Mol Reprod Dev 2000; 57(2): 135–145.
25. Singh R, Fouladi-Nashta AA, Li D et al. Methotrexate induced differentiation in colon cancer cells is primarily due to purine deprivation. J Cell Biochem 2006; 99(1): 146–155.
26. Serra JM, Gutiérrez A, Alemany R et al. Inhibition of c-Myc down-regulation by sustained extracellular signal-regulated kinase activation prevents the antimetabolite methotrexate- and gemcitabine-induced differentiation in non-small-cell lung cancer cells. Mol Pharmacol 2008; 73(6): 1679–1687.
27. Lin TL, Vala MS, Barber JP et al. Induction of acute lymphocytic leukemia differentiation by maintenance therapy. Leukemia 2007; 21(9): 1915–1920.
28. Hatse S, De Clercq E, Balzarini J. Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. Biochem Pharmacol 1999; 58(4): 539–555.
29. Hara A, Niwa M, Kumada M et al. Intraocular injection of folate antagonist methotrexate induces neuronal differentiation of embryonic stem cells transplanted in the adult mouse retina. Brain Res 2006; 1085(1): 33–42.
30. Hara A, Taguchi A, Aoki H et al. Folate antagonist, methotrexate induces neuronal differentiation of human embryonic stem cells transplanted into nude mouse retina. Neurosci Lett 2010; 477(3): 138–143.
31. Stover PJ. One-carbon metabolism-genome interactions in folate-associated pathologies. J Nutr 2009; 139(12): 2402–2405.
32. Vezmar S, Schüsseler P, Becker A et al. Methotrexate-associated alterations of the folate and methyl-transfer pathway in the CSF of ALL patients with and without symptoms of neurotoxicity. Pediatr Blood Cancer 2009; 52(1): 26–32.
33. Li M, Hu SL, Shen ZJ et al. High-performance capillary electrophoretic method for the quantification of global DNA methylation: application to methotrexate-resistant cells. Anal Biochem 2009; 387(1): 71–75.
34. Wang YC, Chiang EP. Low-dose methotrexate inhibits methionine S-adenosyltransferase in vitro and in vivo. Mol Med 2012; 18(1): 423–432.
35. Winter-Vann AM, Kamen BA, Bergo MO et al. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc Natl Acad Sci U S A 2003; 100(11): 6529–6534.
36. Wu J, Wood GS. Reduction of Fas/CD95 promoter methylation, upregulation of Fas protein, and enhancement of sensitivity to apoptosis in cutaneous T-cell lymphoma. Arch Dermatol 2011; 147(4): 443–449.
37. Salbaum JM, Kappen C. Genetic and epigenomic footprints of folate. Prog Mol Biol Transl Sci 2012; 108: 129–158.
38. Mahoney SE, Yao Z, Keyes CC et al. Genome-wide DNA methylation studies suggest distinct DNA methylation patterns in pediatric embryonal and alveolar rhabdomyosarcomas. Epigenetics 2012; 7(4): 400–408.
39. Diede SJ, Guenthoer J, Geng LN et al. DNA methylation of developmental genes in pediatric medulloblastomas identified by denaturation analysis of methylation differences. Proc Natl Acad Sci U S A 2010; 107(1): 234–239.
40. Restrepo A, Smith CA, Agnihotri S et al. Epigenetic regulation of glial fibrillary acidic protein by DNA methylation in human malignant gliomas. Neuro Oncol 2011; 13(1): 42–50.
41. Hill VK, Underhill-Day N, Krex D et al. Epigenetic inactivation of the RASSF10 candidate tumor suppressor gene is a frequent and an early event in gliomagenesis. Oncogene 2011; 30(8): 978–989.
42. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21(35): 5427–5440.
43. Yang PM, Lin JH, Huang WY et al. Inhibition of histone deacetylase activity is a novel function of the antifolate drug methotrexate. Biochem Biophys Res Commun 2010; 391(3): 1396–1399.
44. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 2007; 26(37): 5541–5552.
45. Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. J Cell Biochem 2009; 107(4): 600–608.
46. Huang WY, Yang PM, Chang YF et al. Methotrexate induces apoptosis through p53/p21-dependent pathway and increases E-cadherin expression through downregulation of HDAC/EZH2. Biochem Pharmacol 2011; 81(4): 510–517.
47. Chang CJ, Hung MC. The role of EZH2 in tumour progression. Br J Cancer 2012; 106(2): 243–247.
48. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006; 5(9): 769–784.
49. Dell'Aversana C, Lepore I, Altucci L. HDAC modulation and cell death in the clinic. Exp Cell Res 2012; 318(11): 1229–1244.
50. Leclerc GJ, Mou C, Leclerc GM et al. Histone deacetylase inhibitors induce FPGS mRNA expression and intracellular accumulation of long-chain methotrexate polyglutamates in childhood acute lymphoblastic leukemia: implications for combination therapy. Leukemia 2010; 24(3): 552–562.
51. Einsiedel HG, Kawan L, Eckert C et al. Histone deacetylase inhibitors have antitumor activity in two NOD/SCID mouse models of B-cell precursor childhood acute lymphoblastic leukemia. Leukemia 2006; 20(8): 1435–1436.
52. Bastian L, Einsiedel HG, Henze G et al. The sequence of application of methotrexate and histone deacetylase inhibitors determines either a synergistic or an antagonistic response in childhood acute lymphoblastic leukemia cells. Leukemia 2011; 25(2): 359–361.
53. Prasad P, Vasquez H, Das CM et al. Histone acetylation resulting in resistance to methotrexate in choroid plexus cells. J Neurooncol 2009; 91(3): 279–286.
54. Thornalley PJ, Rabbani N. Glyoxalase in tumourigenesis and multidrug resistance. Semin Cell Dev Biol 2011; 22(3): 318–325.
55. Santarius T, Bignell GR, Greenman CD et al. GLO1-A novel amplified gene in human cancer. Genes Chromosomes Cancer 2010; 49(8): 711–725.

56. Suji G, Sivakami S. DNA damage during glycation of lysine by methylglyoxal: assessment of vitamins in preventing damage. Amino Acids 2007; 33(4): 615–621.
57. Pepper ED, Farrell MJ, Nord G et al. Antiglycation effects of carnosine and other compounds on the long--term survival of Escherichia coli. Appl Environ Microbiol 2010; 76(24): 7925–7930.
58. Kalapos MP. The tandem of free radicals and methylglyoxal. Chem Biol Interact 2008; 171(3): 251–271.
59. Koizumi K, Nakayama M, Zhu WJ et al. Characteristic effects of methylglyoxal and its degraded product formate on viability of human histiocytes: a possible detoxification pathway of methylglyoxal. Biochem Biophys Res Commun 2011; 407(2): 426–431.
60. Uzar E, Koyuncuoglu HR, Uz E et al. The activities of antioxidant enzymes and the level of malondialdehyde in cerebellum of rats subjected to methotrexate: protective effect of caffeic acid phenethyl ester. Mol Cell Biochem 2006; 291(1–2): 63–68.
61. Miketova P, Kaemingk K, Hockenberry M et al. Oxidative changes in cerebral spinal fluid phosphatidylcholine during treatment for acute lymphoblastic leukemia. Biol Res Nurs 2005; 6(3): 187–195.
62. Jahovic N, Cevik H, Sehirli AO et al. Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. J Pineal Res 2003; 34(4): 282–287.
63. Caetano NN, Campello AP, Carnieri EG et al. Effect of methotrexate (MTX) on NAD(P)+ dehydrogenases of HeLa cells: malic enzyme, 2-oxoglutarate and isocitrate dehydrogenases. Cell Biochem Funct 1997; 15(4): 259–264.
64. Babiak RM, Campello AP, Carnieri EG et al. Methotrexate: pentose cycle and oxidative stress. Cell Biochem Funct 1998; 16(4): 283–293.
65. Vardi N, Parlakpinar H, Ates B. Beneficial effects of chlorogenic acid on methotrexate-induced cerebellar Purkinje cell damage in rats. J Chem Neuroanat 2012; 43(1): 43–47.
66. Spurlock CF 3rd, Aune ZT, Tossberg JT et al. Increased sensitivity to apoptosis induced by methotrexate is mediated by JNK. Arthritis Rheum 2011; 63(9): 2606–2616.
67. Zapletalova D, André N, Deak L et al. Metronomic chemotherapy with the COMBAT regimen in advanced pediatric malignancies: a multicenter experience. Oncology 2012; 82(5): 249–260.
68. Carrasco MP, Enyedy EA, Krupenko NI et al. Novel folate-hydroxamate based antimetabolites: synthesis and biological evaluation. Med Chem 2011; 7(4): 265–274.
69. Zimmermann GR, Lehár J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 2007; 12(1–2): 34–42.