Multi-targeted Antifolates - Whole Cell Assay

Inhibition of *de novo* purine and pyrimidine biosynthesis

IMPORTANT: Client-specified alterations can be accommodated.

To study the effect of nucleoside analogues on the whole spectra of cellular purine and pyrimidine ribo- and deoxyribonucleotides, we have developed an original cell-based analytical approach in which more than 31 (deoxy)ribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pared chromatography and quantified. These cellular assays were validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, hydroxyurea, methotrexate).

Aim

"Multi-targeted Antifolates: Whole Cell Assay" has been specially tailored to validate inhibition of *de novo* biosynthesis of purine and pyrimidne nucleotides by a given compound in cultured cells. After incubation of cultured cells with the inhibitor, nucleotides are extracted, separated, identified and quantified by UV-HPLC. This service was validated with methotrexate in HeLa cultured cells.

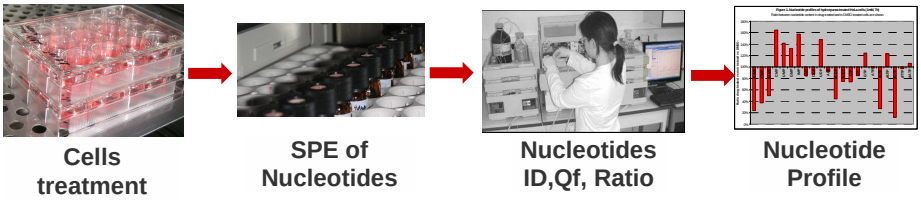
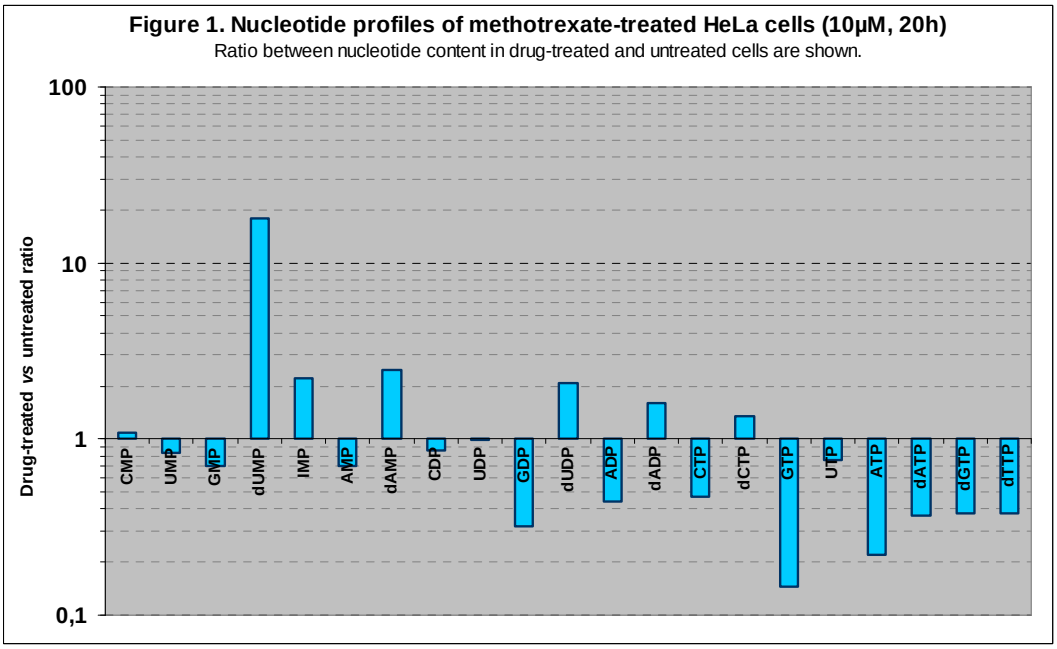


Figure 1. Nucleotide profiles of methotrexate-treated HeLa cells (10µM, 20h)
Ratio between nucleotide content in drug-treated and untreated cells are shown.



Methotrexate (MTX) is an immunosuppressive agent that has been in clinical use for over 50 years. Although originally introduced for chemotherapy in cancer and leukaemia, MTX was coincidentally found to have immunosuppressive properties and is currently used in treating rheumatoid arthritis[1]. MTX was first believed to be an inhibitor of the enzyme dihydrofolate reductase (DHFR), the enzyme required for reduction of dihydrofolate (FH_2) to tetrahydrolate (FH_4). However, as shown in the 80's, MTX is actually a prodrug which is polyglutamated and accumulated in cells[2]. In contrast to unmodified MTX, its polyglutamated derivatives were found to be efficient inhibitors of the ninth folate-dependent step of purine synthesis catalysed by 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate transformylase (AICAR-T)[3] and the thymidylate synthase (TS)[4].

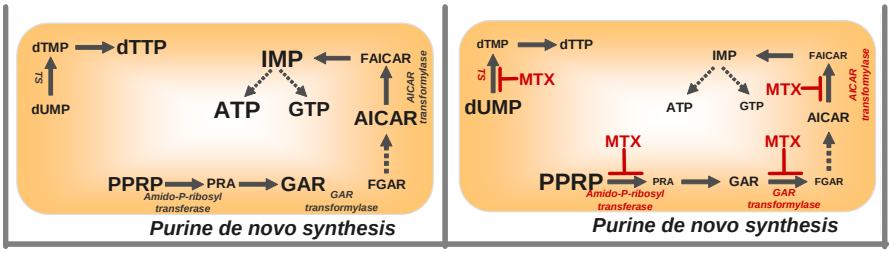


Figure 2. Effect of MTX on cellular pool of purines and pyrimidines.

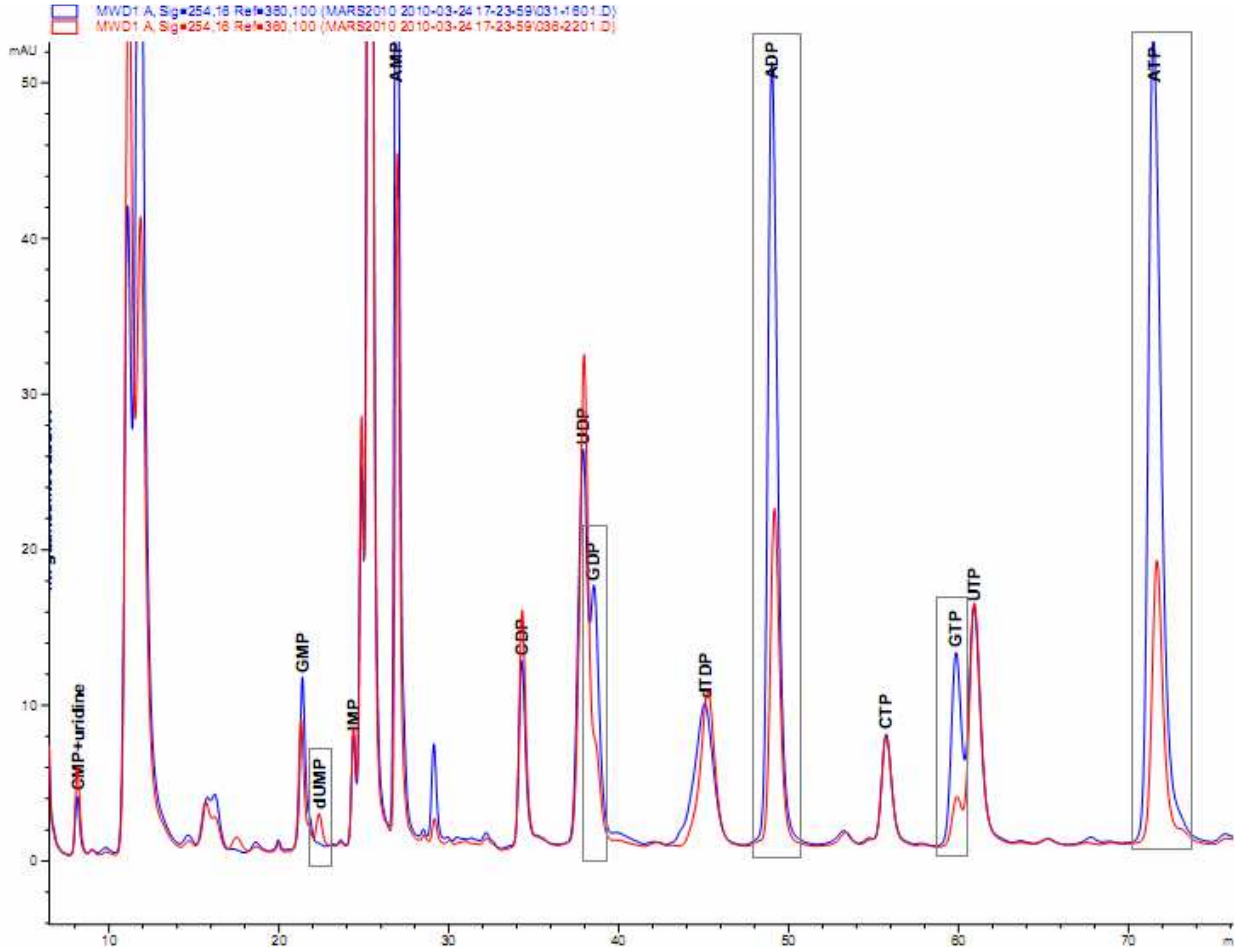


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 10µM MTX (red) and DMSO (blue). The specific depletion in purine nucleotides, *e.g.* ATP, ADP, GTP and GDP, and accumulation of dUMP, as a result of TS inhibition, are framed in grey.

Results:

As illustrated by Figures 1 and 3, intracellular level of ATP, ADP, GTP and GDP is much lower in methotrexate-treated cells than in untreated control, while cellular contents of UTP and UDP are not affected. Another remarkable change concerns the accumulation of dUMP in methotrexate-treated HeLa cell and depletion of dTTP pool. All these results are in perfect agreement with previously published data[5] showing that MTX inhibits *de novo* synthesis of purine nucleotides through AICART enzyme and synthesis of thymidylate through thymidilate synthase.

Materials & Methods

Cells treatment: HeLa cells were grown in an atmosphere of humidified 5% CO_2 at 37°C in DMEM (PAA) medium supplemented with 2mM L-glutamine (Gibco/BRL), non essential amino acids (PAA), 10% heat-inactivated fetal bovine serum (BioWest) and streptomycin-penicillin (Sigma). Exponentially grown HeLa cells were seeded at ~$6x10^5$ cells per dish. After 48h of growth, the culture medium was replaced with fresh FCS-supplemented medium (10ml per Petri dish) followed by addition of 10µL of DMSO or DMSO-dissolved compounds. Six Petri dishes of cells per experiment were used to provide the nucleotide amount sufficient for UV-quantification of deoxynucleotides. At the end of a 7h-incubation, the medium was aspirated, cells monolayers washed twice with 5ml PBS, and used for nucleotides extraction.

Extraction of nucleotides and deoxynucleotides - Sample preparation: The nucleotides were extracted from cell monolayers by the addition of ice-cold 80% acetonitrile for 1h. The extracts were centrifuged to remove cellular debris and load on SAX column (100mg, Supelco) pre-conditioned with methanol, water and acetonitrile. Once the sample was completely effused, the cartridge was washed with 3ml 80% ACN and 3ml water and eluted with 1M KCl. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system: 1) Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and multiple wavelength and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).
5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications as follows.
Peak identification of the different nucleoside mono-, di-, and triphosphates, was made from their characteristic UV absorption spectra and retention times compared with those of a mixture of standards (Sigma) run immediately before the cell extracts. The area of individual peaks was measured using ChemStation software (Agilent).

HPLC conditions: Nucleotides were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides[6] with slight modifications in pH and concentration of buffers adjusted to ensure adequate resolution of all nucleosides/nucleotides as follows: Buffer A: 20mM KH_2PO_4, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH_2PO_4, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature kept constant at 21°C. Gradient was formed as follo ws: 15 min at 100% buffer A; 5 min up to 90% buffer A, 5 min up to 70% buffer A; 15 min up to 63% buffer A, 15 min up to 55% buffer A, 20 min up to 45% buffer A, 10 min up to 25% buffer A, 10 min up to 0% buffer A. The spectra were recorded at 254 and 280nm.

HPLC calibration: The calibration was performed with the following standards: dUMP, dUDP, dUTP, dCDP, dCMP, dCDP, dCTP, dTMP, dTTP. dGDP and dGTP were not separated from unknown major peak and were not quantified. The standards prepared in Buffer A or those mixed with cell extracts were run immediately before and after series of samples. The data were used for calculation of retention times (Rf) and absorbance at 254nm and 280nm (254/280 ratio), specific for each nucleotide.

Peak identification and quantification: 5µl of cell extract were injected and nucleotides were separated as described before. Assignment of peak of the different deoxyribonucleosides and ribonucleosides mono-, di-, and triphosphate was done by comparing both retention times and characteristic UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

Quality control: The experiments are done in duplicates and relative standard deviation (RSD) is usually less than 12%.

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