



Whole cell analytical assay for targeting purine and pyrimidine biosynthesis: Application for discovery of new anti-proliferative immunosuppressive drugs

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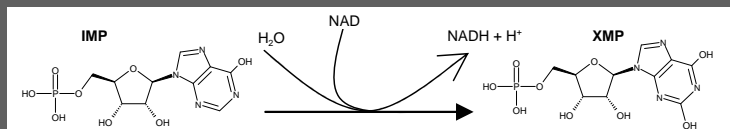
ABSTRACT

The depletion of T- and B-lymphocytes due to genetic disorders of purine and pyrimidine metabolism highlights the importance of these pathways to immune response. The therapeutic potential of targeting enzymes of the nucleotide biosynthesis was displayed by successful application of anti-metabolite drugs in the treatment of psoriasis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA, e.g. Arava® - leflunomide, inhibitor of dihydroorotate dehydrogenase DHODH) and to prevent transplant rejection (CellCept® - a prodrug of mycophenolic acid MPA, inhibitor of inosine monophosphate dehydrogenase IMPDH).

- i) We have cloned a human recombinant IMPDH II and developed *in vitro* enzymatic assays for search of new IMPDH inhibitors.
- ii) We have developed a whole cell assay to select compounds that are able to penetrate cells and target the intracellular IMPDH.
- iii) We have developed whole cell analytical assays, based on separation, identification and quantification by HPLC of complete spectra of nucleosides and nucleotides to study the effect of drugs on nucleoside metabolism.

IMPDH, a choice target for the treatment of immunological diseases

Inosine Monophosphate Dehydrogenase (IMPDH) converts inosine 5'-monophosphate (IMP) to xanthosine 5'- monophosphate (XMP) using NAD+ as a cofactor.

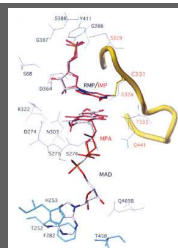
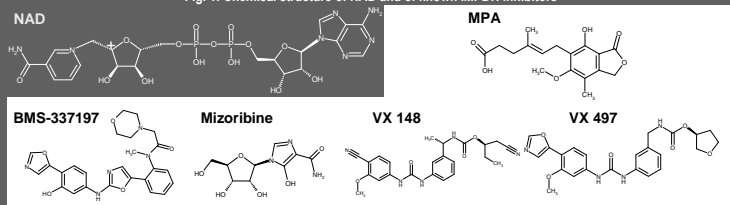


The oxidation of IMP to XMP is considered as the pivotal step in the biosynthesis of guanine nucleotides[1] whose decrease resulting from IMPDH inhibition interrupts the nucleic acid synthesis in proliferating cells. IMPDH Type II is the predominant isoform of the enzyme and is selectively expressed in lymphocytes and tumor cells[1].

IMPDH is considered as a potent target for cancer chemotherapy[1] (e.g. AVN-944) and for the treatment of autoimmune diseases such as psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[2,3]. CellCept® - a mycophenolic acid (MPA) prodrug -, is a successful immunosuppressive agent (CHF1.85 bn in 2006) used to prevent transplant rejection. The extension of CellCept® therapeutic applications (orphan drug for Myasthenia gravis, phase III for SLE) makes IMPDH an attractive validated target to treat numerous immunological diseases.

Besides MPA analogues and nucleosides analogues (ribavirin - Virazole®, Rebeto®, Copegus® -, mizoribine - Bredinin® and tiazofurin - Tiazole), other NCEs have been identified as IMPDH inhibitors[2,4,5] and entered development trials.

Fig. 1: Chemical structure of NAD and of known IMPDH inhibitors



The IMPDH II atomic structure has been resolved and it provides a valuable background for further leads optimization.

All this demonstrates how promising the research of new IMPDH inhibitors is and why compounds are worth being evaluated on such a highly pertinent target.

Whole cell analytical assay for nucleotide biosynthesis inhibition

In order to:

- confirm the ability of IMPDH inhibitors *in vitro* selected to decrease the cellular pool of GTP;
 - estimate the intracellular IC₅₀;
 - evaluate the compounds specificity by comparing GTP, ATP, UTP and CTP cell contents;
- we have developed a whole cell bio-analytical assay that consists in separating and quantifying nucleotides extracted from drug treated cells. The cell assay has been validated using known inhibitors of purine and pyrimidine biosynthesis: MPA, which targets IMP dehydrogenase, and leflunomide (LFM), which acts on dihydroorotate dehydrogenase (DHODH). According to previously published data, MPA inhibits the formation of guanosine nucleotides, whereas LFM decreases the level of uridine nucleotides.

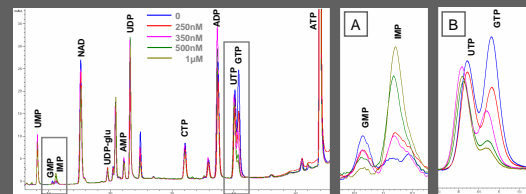
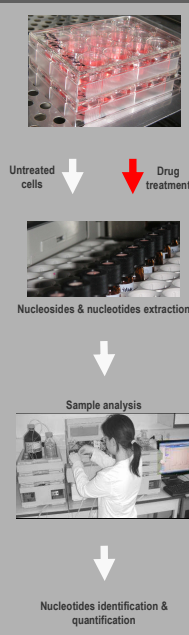


Fig. 3: Chromatograms of TCA-extracts of cultured Huh-7 cells treated with increasing concentrations of MPA for 48h. The nucleoside contents of cells extracts were analyzed using HPLC Agilent 1100 fitted up with autosampler and variable wavelength detector. The nucleosides are separated using a Zorbax EclipsePlus C18 column. A: focus on GMP and IMP B: focus on UTP and GTP

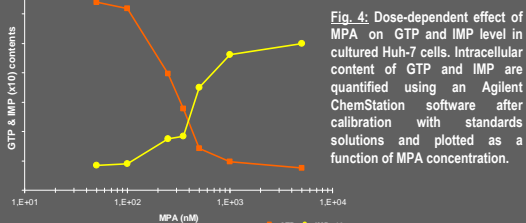


Fig. 4: Dose-dependent effect of MPA on GTP and IMP level in cultured Huh-7 cells. Intracellular content of GTP and IMP are quantified using an Agilent ChemStation software after calibration with standards solutions and plotted as a function of MPA concentration.

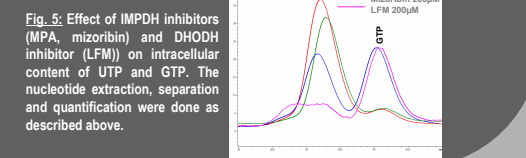


Fig. 5: Effect of IMPDH inhibitors (MPA, mizoribin) and DHODH inhibitor (LFM) on intracellular content of UTP and GTP. The nucleotide extraction, separation and quantification were done as described above.

Enzymatic characterization of human IMPDH II

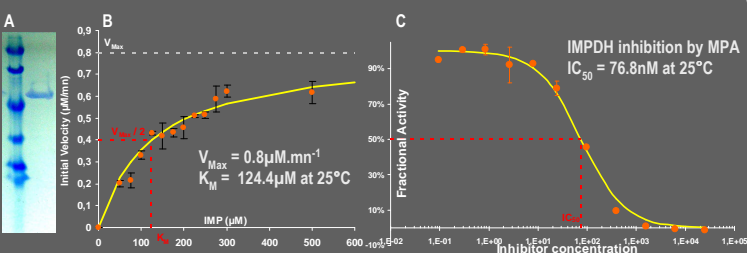


Fig. 2: (A) The cDNA encoding human IMPDH II was cloned by RT-PCR amplification of RNA from human hepatocarcinoma Huh7 in fusion with C-terminal His-tag. The IMPDH II protein was overexpressed in *E.coli* and purified on Ni-NTA agarose (Qiagen). (B) The enzyme activity was spectrophotometrically determined at 25°C by monitoring the formation of NADH at 340nm with an iEMS plate reader (Labsystems). (C) IC₅₀ value was measured at 180µM NAD and 100µM IMP. The fractional activity - ratio between the maximal activity observed (i.e. without inhibitor) and the activity at each compound concentration - is plotted as a function of inhibitor concentration. IC₅₀ is then calculated using a standard four-parameter non-linear regression analysis.

Conclusions

We have established and validated:

1. an *in vitro* enzymatic assay for the identification of new IMPDH II inhibitors
2. a whole cell based assay to select compounds that are able to penetrate cells and reach intracellular IMPDH.
3. a bio-analytical method for the search of inhibitors of the purine and pyrimidine biosynthesis, which is considered as a rich source of targets for immunosuppression or anti-cancer treatment.

References

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