Development of a targeted HILIC-MRM Method for the Quantification of Tri*PPP*ro Prodrugs and their Metabolites in complex Mixtures

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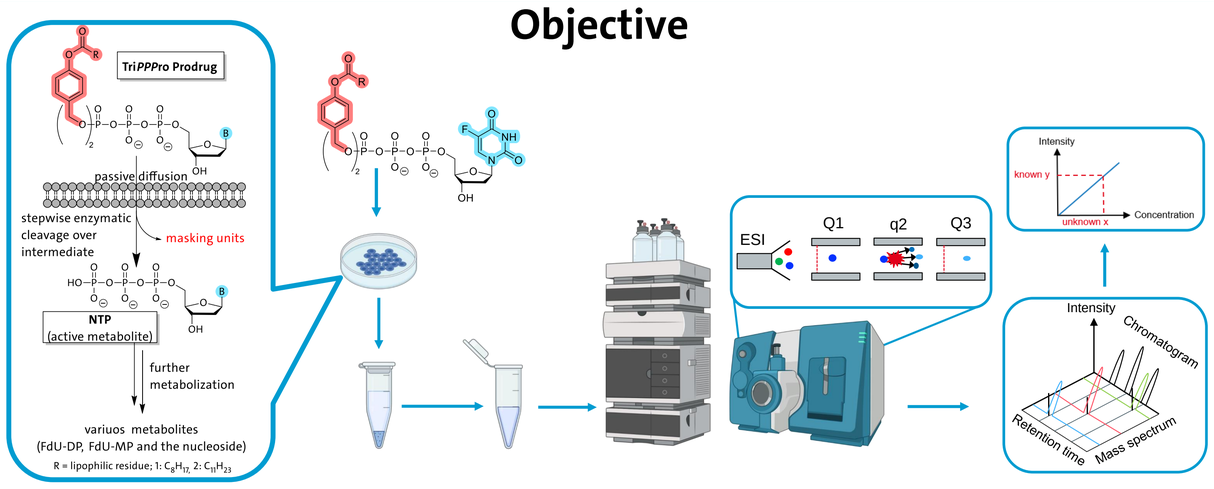
Background

Introduction

- Nucleoside and nucleotide analogues in anticancer and antiviral chemotherapy
  → frontline of drugs to combat infections caused by several viruses
- Nucleoside analogue drugs must be metabolized by host cell kinases to undergo stepwise phosphorylation yielding bioactive nucleoside triphosphate (NTP) → this conversion often proceeds insufficient
- To directly deliver triphosphate metabolites: we have developed the Tri*PPP*ro-approach[1]
  → γ-phosphate of NTP is masked by two lipophilic biocleavable units and is therefore able to penetrate cell membrane; after enzymatic cleavage of masks: bioactive NTPs are released.
- To demonstrate successful uptake and intracellular delivery of metabolites: cell uptake studies are performed and analyzed by LC-MS/MS

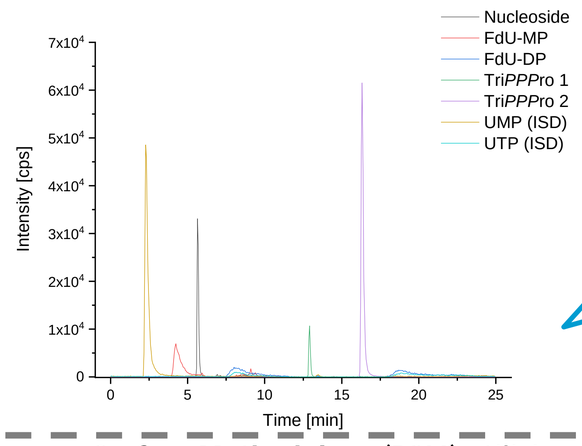
Objective



Results

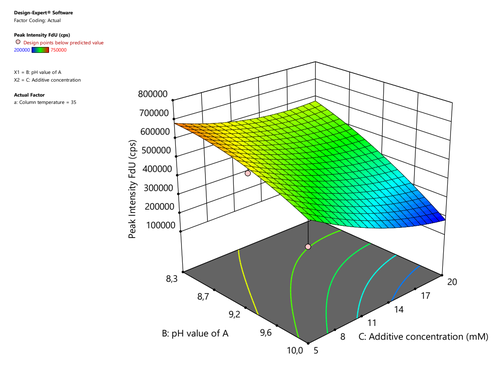
1 – Initial MS tuning & chromatographic development

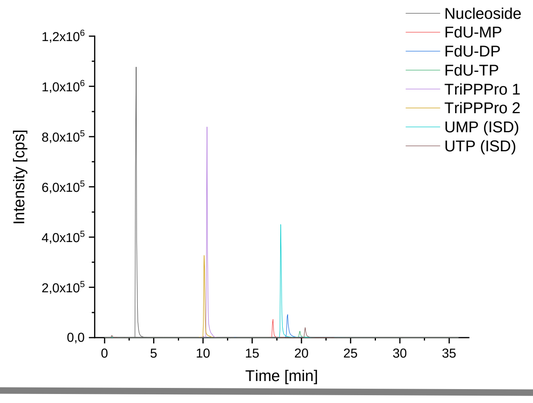
- Identifying precursor ions (Q1) and three different fragment ions (Q3) for each analyte on a SCIEX QTRAP 5500 (ESI in negative mode)
- Development of a **Multiple Reaction Monitoring** (MRM) method with characteristic mass transitions (Q1/Q3)
- Tri*PPP*ro prodrugs and metabolites showed **different chemical properties**, therefore major challenge to achieve simultaneous retention within one LC run



- Preliminary tests of the LC conditions:
- Unable to quantify most peaks due to peak splitting and broadening
- NTP did not show any signal

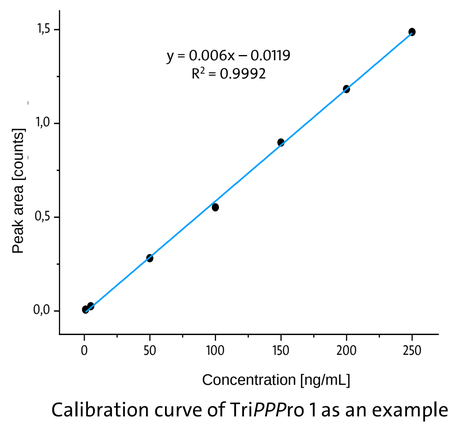
- **Response Surface Methodology** (RSM) utilizing design of experiments to optimize parameters for LC method
- Design of a ternary gradient using **HILIC conditions** allows simultaneous retention of all analytes and internal standards (ISD) within a single LC run





2 – MS optimization & linearity / sensitivity

- MRM parameters optimized using **Flow Injection Analysis** (FIA) for higher signal intensity
- Evaluation of linear range with ISD with calibration standards (CS) prepared in cell lysate

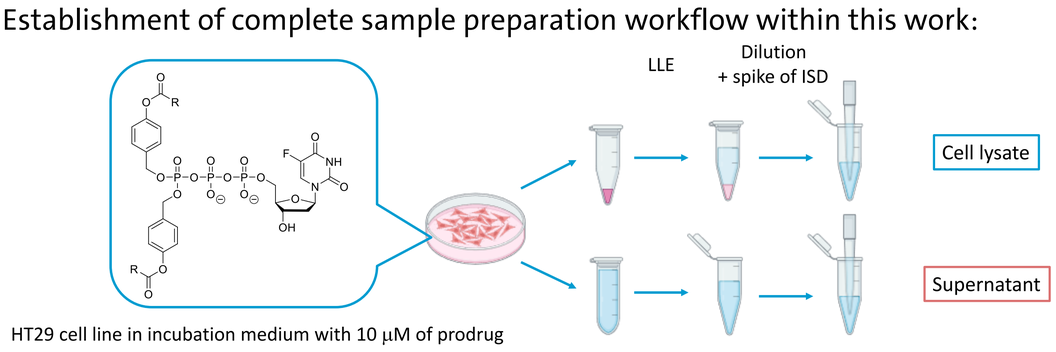


Calibration curve of Tri*PPP*ro 1 as an example

Compound	LOD [ng/mL]	LOQ [ng/mL]
Nucleoside	2.66	8.88
Tri*PPP*ro 1	35.8	119
Tri*PPP*ro 2	2.07	6.92
Intermediate 1	31.2	104
Intermediate 2	4.71	15.7
FdU-MP	10.1	33.7
FdU-DP	20.3	67.7
FdU-TP	28.0	93.4

3 – Extraction & matrix effect / recovery rate

- Establishment of complete sample preparation workflow within this work:



HT29 cell line in incubation medium with 10 µM of prodrug

- After cell uptake, cell pellets were lysed followed by **Liquid-Liquid-Extraction** (LLE) using MTBE:MeOH:H_2O (10:3:2.5, % *v/v/v*) with 5% phosphatase inhibitor

Compound	Matrix effect [%]	Recovery [%]
Nucleoside	-17	76
Tri*PPP*ro 1	76	28
Tri*PPP*ro 2	37	45
Intermediate 1	-17	115
Intermediate 2	-63	121
FdU-MP	-54	N/A
FdU-DP	-31	87
FdU-TP	-3	44

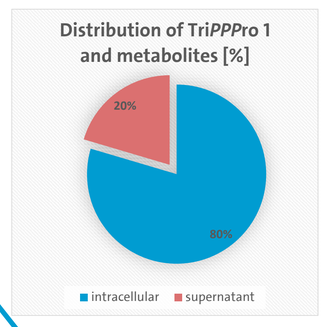
4 – Quantification results

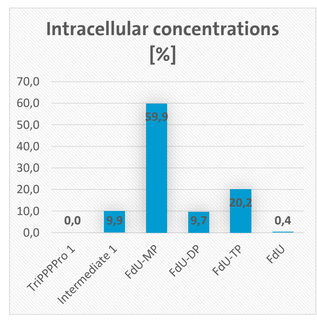
- Results of **cell uptake study** of Tri*PPP*ro 1 and Tri*PPP*ro 2 in HT29 cancer cell lines (n = 2)

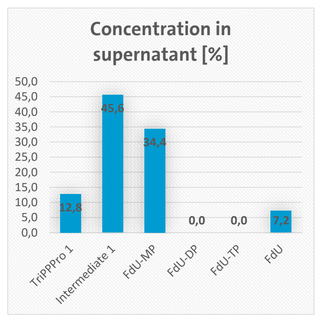
Compound	Concentration in cell lysate [µM]	Concentration in supernatant [µM]
Nucleoside	0.03	0.09
Tri*PPP*ro 1	ND	0.16
Intermediate 1	0.83	0.57
FdU-MP	5.01	0.43
FdU-DP	0.81	ND
FdU-TP	1.69	ND

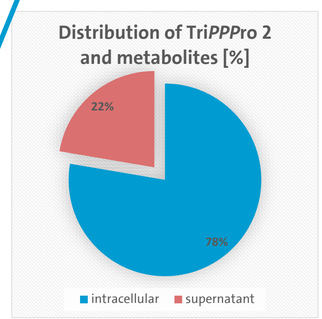
Compound	Concentration in cell lysate [µM]	Concentration in supernatant [µM]
Nucleoside	0.02	0.02
Tri*PPP*ro 2	0.40	3.13
Intermediate 2	1.85	0.13
FdU-MP	8.82	0.26
FdU-DP	0.12	ND
FdU-TP	0.25	ND

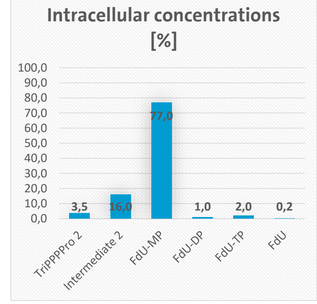
- For both Prodrugs: cell uptake very successful
- Intracellular formation of the bioactive FdU-MP

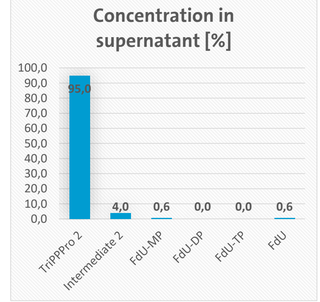












Conclusion

- Effective HILIC-MS/MS method was developed for **simultaneous analysis** of Tri*PPP*ro prodrugs and their metabolites in complex mixtures
- Optimization of LC conditions (e.g. additive concentration) with **RSM** led to robust method
- Establishment of an **LLE-protocol** to optimize sample preparation
- Potential uses of this method include investigation of lipophilic prodrug uptake efficiency and insides into intracellular drug metabolism

Future work

- Investigation of the same antitumoral Tri*PPP*ro prodrugs in different cancer cell line (SW620) to compare cell uptake
- Quantification of different antiviral prodrugs (e.g. d4T derivatives) in other cell lines (CEM/0)
- Exploring possibilities for method transfer to other phosphate bearing molecules, not only Tri*PPP*ro compounds (e.g. ADPR or NAADP derivatives)

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References

[1] T. Gollnest, T. D. De Oliveira, A. Rath, I. Hauber, D. Schols, J. Balzarini, C. Meier, *Angew. Chem. Int. Ed.* **2016**, *55*, 5255–5258.