Analytical Mass Spectrometry Method for Quantification of Tri*PPP*ro-Prodrugs and their Metabolites in Cell Extracts

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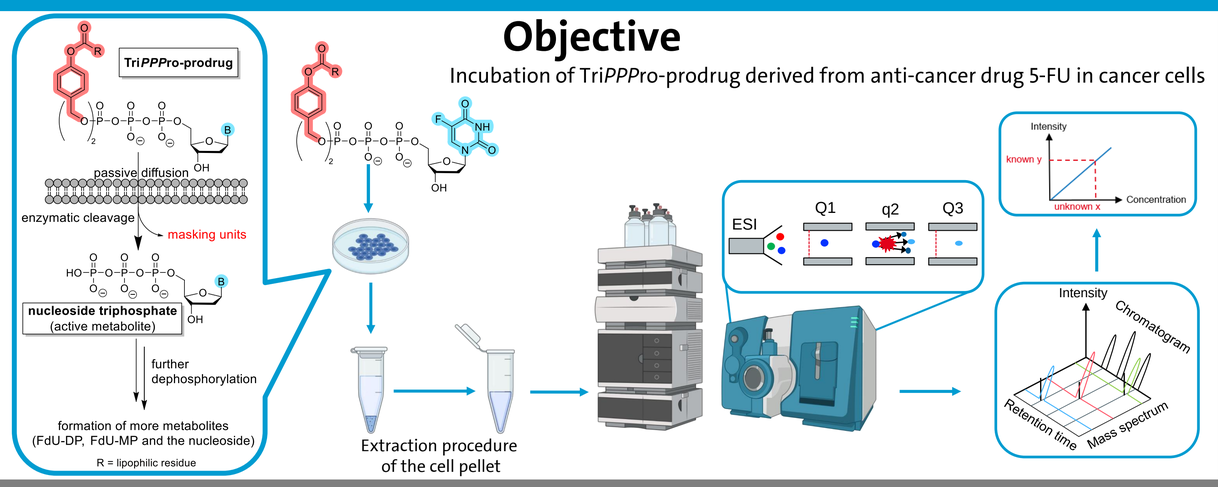
Background

Introduction

Nucleoside and nucleotide analogues are used in anticancer and antiviral chemotherapy and belong to one of the most important groups of drugs used to combat viral infections. In general, nucleoside analogues must be metabolized and stepwise phosphorylated in the host cell to yield the active nucleoside triphosphate (NTP) analogue. This metabolic conversion is often slow and inefficient. To deliver the active metabolites directly, our group has developed the Tri*PPP*ro approach. In this technology, the γ-phosphate of an NTP analogue is masked by two lipophilic moieties, allowing cell membrane penetration. After enzymatic cleavage, the bioactive NTP is formed. Qualitative cell uptake studies were performed to investigate the successful uptake and intracellular release of the metabolites.[1]

Objective

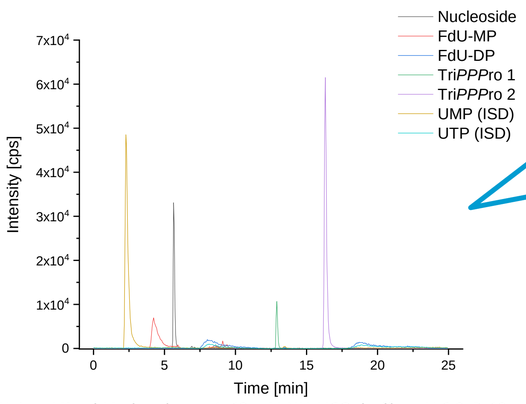
Incubation of Tri*PPP*ro-prodrug derived from anti-cancer drug 5-FU in cancer cells



Results

1 – Initial MS tuning & chromatography development

- Identifying precursor ions (Q1) and three different fragment ions (Q3) for every analyte of interest on a SCIEX QTRAP 5500 using electrospray ionization (ESI) in negative mode
- Development of a *Multiple Reaction Monitoring* (MRM) method with characteristic mass transitions
- Tri*PPP*ro-prodrugs and resulting nucleotides & nucleosides show very different chemical properties: simultaneous retention as major challenge



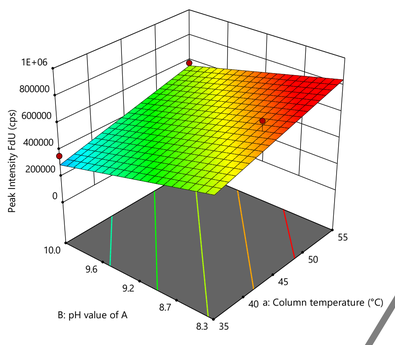
- Unable to quantify most peaks due to peak splitting and broadening
- NTP did not show any signal

Waters Mixed Mode column, A: 10 mM NH_4HCO_3 buffer, pH 6.0, B: 10 mM NH_4HCO_3 in MeCN/IPA/H_2O, 500 ng/mL in 100% water

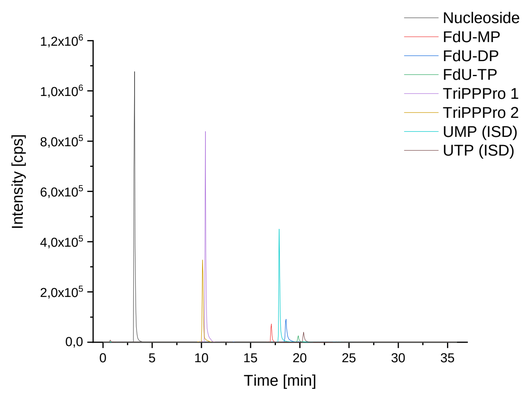
- *Response Surface Methodology* (RSM) utilizing design of experiments was used to optimize the parameters for the LC method

Optimal conditions:

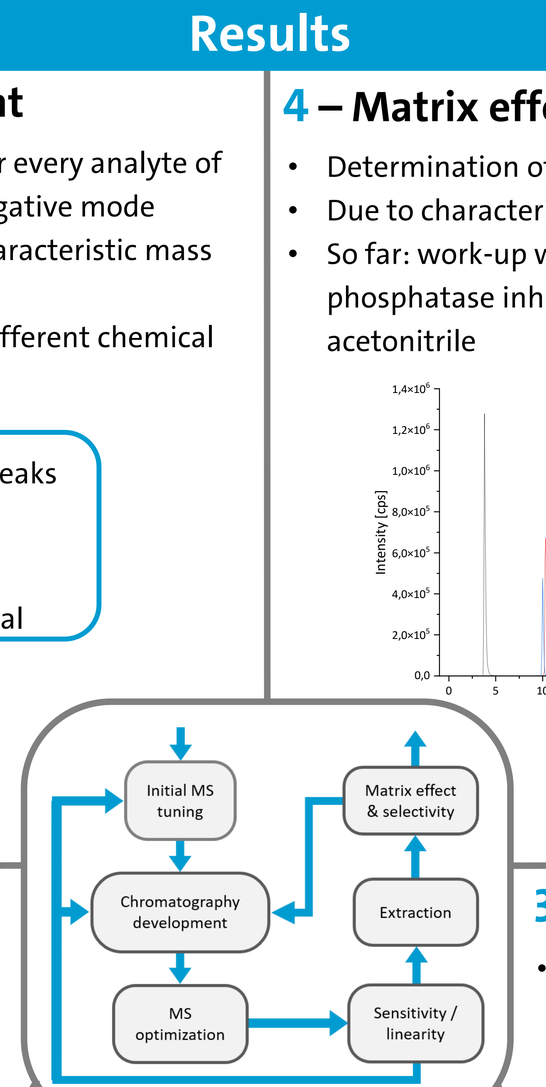
- pH value of the buffered mobile phase = 8.3
- Additive concentration for mobile phase = 5 mM
- Column temperature = 55 °C



- Design of a ternary gradient using HILIC conditions allows simultaneous retention of all analytes and internal standards (ISD)



Waters Amide column, A: 100 mM NH_4HCO_3 buffer, pH 8.3, B: MeCN, C: H_2O, 500 ng/mL in 95/5 (v/v %) MeCN/H_2O

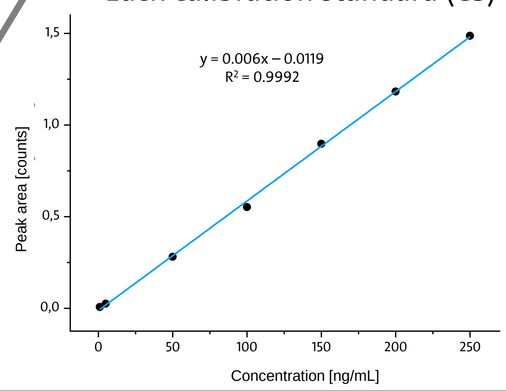


2 – MS optimization & sensitivity / linearity

- MRM parameters were optimized using *Flow Injection Analysis* (FIA) for higher signal intensity

Compound	Q1 (Da)	Q3 (Da)	Adduct	DP (V)	EP (V)	CE (V)	CXP (V)
Tri*PPP*ro 1	977.1	78.9	$[M-H]^-$	-60.0	-10.0	-140.0	-12.0

- Evaluation of linear range of every analyte with ISD
- Each calibration standard (CS) was prepared in cell lysate

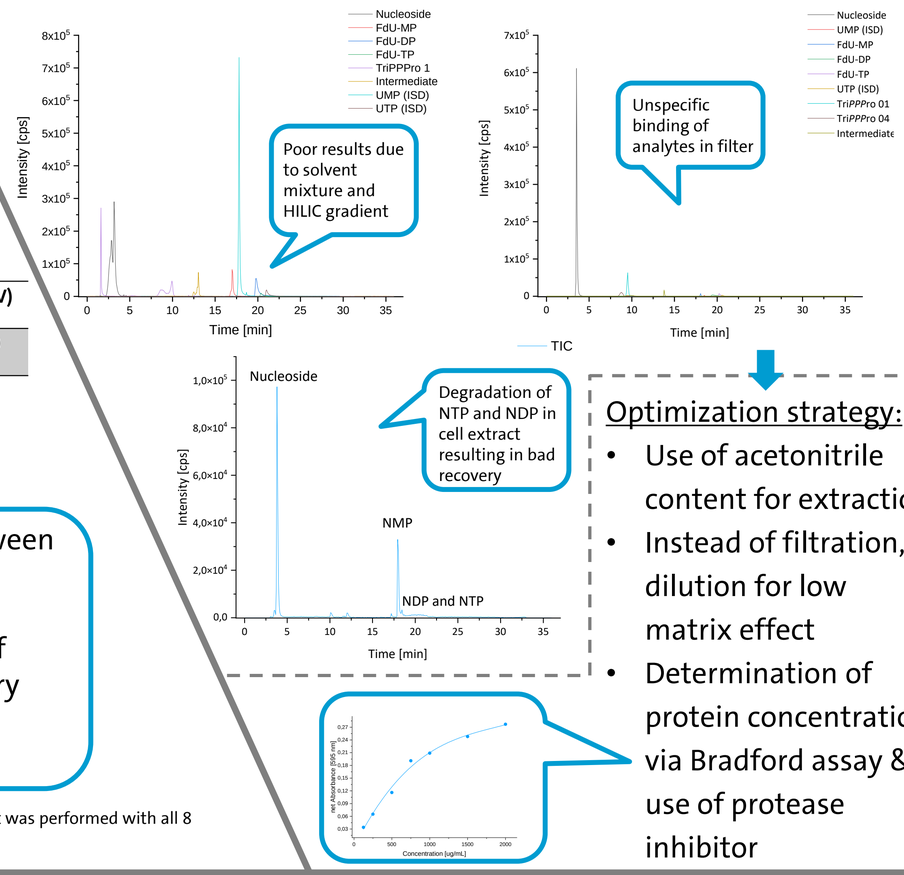


- Linear range between 1.0 ng/mL and 250 ng/mL
- Amount of 5 pg of substance still very well detectable (fmol range)

Shown exemplarily on one compound, but was performed with all 8 analytes from the set

3 – Extraction

- After cell uptake studies, cell pellets were lysed, protein precipitation with 2:1 (v/v %) MeOH/H_2O and subsequent centrifugation and filtration was carried out

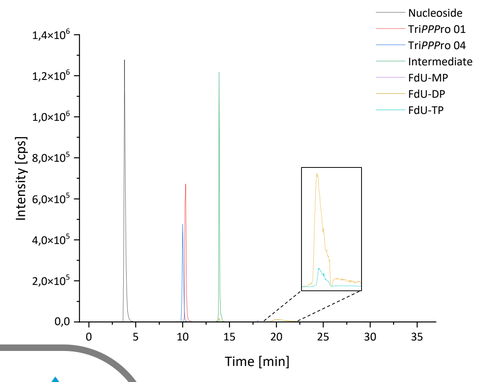


Optimization strategy:

- Use of acetonitrile content for extraction
- Instead of filtration, dilution for low matrix effect
- Determination of protein concentration via Bradford assay & use of protease inhibitor

4 – Matrix effect & selectivity

- Determination of matrix effect in SW620 (human colorectal adeno-carcinoma) cell line
- Due to characteristic mass transition for each analyte: high selectivity in matrix
- So far: work-up with a mixture of MeOH/H_2O for protein precipitation, addition of phosphatase inhibitor cocktail, centrifugation and subsequent dilution of lysate with acetonitrile



Compound	Matrix effect [%]	Recovery rate [%]
Nucleoside	38	64
Tri*PPP*ro 01	51	81
Tri*PPP*ro 02	50	14
Intermediate 01*	41	71
FdU-MP	229	20
FdU-DP	166	18
FdU-TP	101	17

* Intermediate describes a compound with one lipophilic mask cleaved off; so far no data for the intermediate associated with the other Tri*PPP*ro-prodrug

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
- Very good ranges in terms of linearity and sensitivity for all compounds
- Elaborated strategies to overcome the challenges related to the extraction procedure

Future work

- Ongoing optimization of extraction protocol to improve values for matrix effect and recovery rates of the analytes
- Finalize validation and quantification of various Tri*PPP*ro-prodrugs and metabolites
- Potential use of this method include the investigation of the efficiency of the Tri*PPP*ro-prodrug delivery concept not only for the designed FdU-prodrugs but also for other antitumoral and antiviral nucleotide prodrugs

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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*, 5255–525