Pull-down assay as a novel approach for the identification of compounds interfering with thyroid hormone signalling in complex environmental mixtures

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BACKGROUND

- Environment is contaminated with **complex mixtures of chemicals,** which frequently elicit **endocrine disrupting (ED) potential,** but its **effect drivers** are mostly **unknown**
- **Thyroid hormone (TH) disruption** can occur through various mechanisms
- Sensitive target is the interference with TH transport (**transthyretin (TTR)** binding), which can result e.g. in **changes in basal metabolism, disruption in early development and neurodevelopment**
- Tools for the identification of ED chemicals in complex mixtures: effect directed analyses (EDA); non/target screening; QSAR

OBJECTIVES

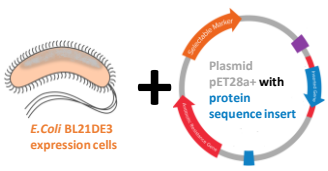
- **To establish and optimize pull-down assay** – promising **tool for separation and identification** of endocrine disruptive chemicals from complex environmental mixtures
- To identify effect drivers of TTR inhibition

METHODS-PRINCIPLE => OUTPUTS

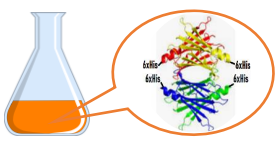
- **Human TTR protein engineering, expression and purification** => **fully functional hTTR** (Fig.1)
- **TTR binding assay**[1] => **preselection** of samples suitable for pull-down assay (passive water extract from a small stream, CZ; (fig.2)); hTTR **functionality** and identified ligands **affinity confirmation** (Tab. 1)
- **Pull-down assay** [2] molecular binding of the compounds into the binding site of the hTTR protein (**prey:bite**) => **separation of** the chemicals specifically bound to hTTR **protein**
- **Non-target MS analysis** [3] – workflow based on HRMS full scan combined with data independent analysis (ESI+ ESI-) => **identification of known/novel hTTR ligands** (Fig.3; Fig.4)
- **Target MS analysis** and **TTR binding assay** – confirmation of known and novel ligands with available analytical standards

Protein engineering, expression and purification

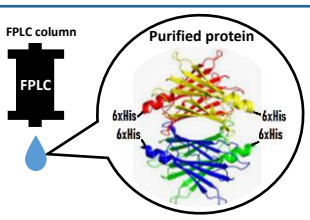
STEP1: Bioinformatic analysis
His-tag added (N-terminal of protein sequence)



STEP2: Transformation of plasmid into *E.coli*

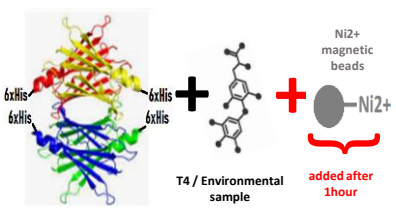


STEP3: Cell propagation and protein expression

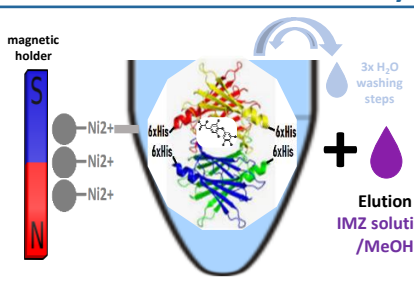


STEP4: FPLC protein purification – for PULL-DOWN ASSAY

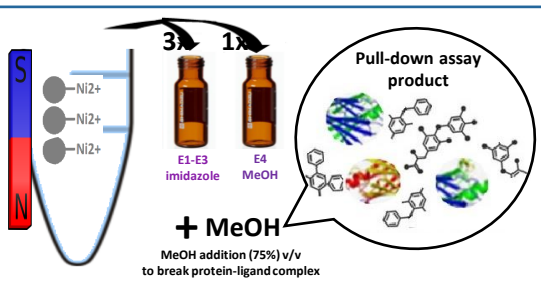
Pull-down assay



STEP5: Protein + ligand (T4)/environmental sample extract incubation and resin beads addition (His-tag binding to Ni^{2+} of the resin beads)

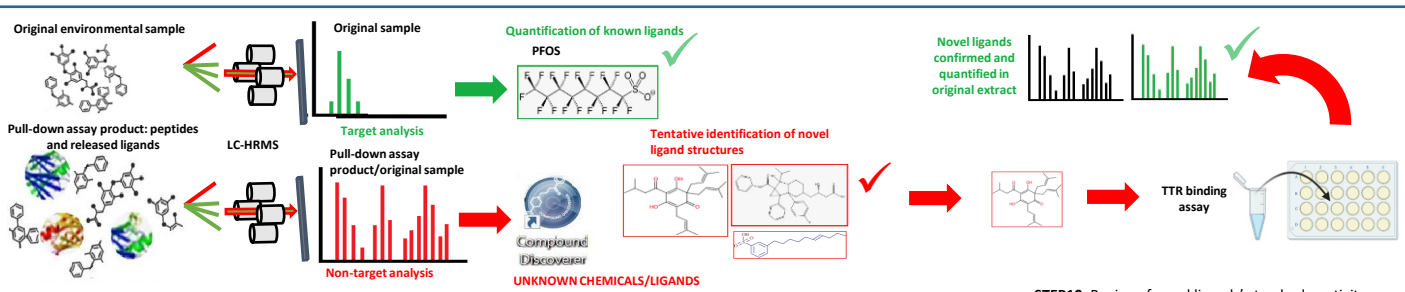


STEP6: Protein-ligand complex attached to Eppendorf tube walls (magnetic holder); unbound contaminants eluted via washing steps => imidazole (IMZ)/ methanol (MeOH) elution



STEP7: Four serial elutions with IMZ: E1-E3; MeOH: E4

Target and Non-target LC-HRMS analysis



STEP8: LC-HRMS target and non-target analysis of original environmental extracts and pull-down products

STEP9: Identification of known ligands in target analysis and tentative identification of novel ligand structures in non-target screening in a workflow based on Compound Discoverer software

UNKNOWN CHEMICALS/LIGANDS

STEP10: Buying of novel ligands' standards, activity confirmation in TTR binding assay and quantification in target LC-HRMS analysis

Results

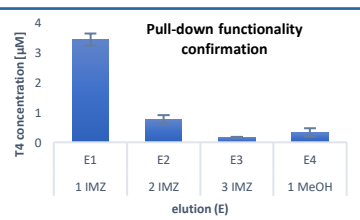


Fig.1 Chemical analysis of T4 (standard ligand) profile bound in pull-down assay

2 localities tested:
WWTP Effluent/ Downstream

Fig.2 Živný Stream

Tab.1 TTR binding inhibition assay - recovery of the effect after pull down assay for Živný stream samples

Bioanalytical equivalent - BEQ-T4 [ug/L]		
	Effluent	**Downstream**
Before pull-down	**22.9±2.9**	**5±1.0**
After pull down	**1.7±0.5**	**0.7±0.4**
Effect recovery %	**7.5**	**13.4**

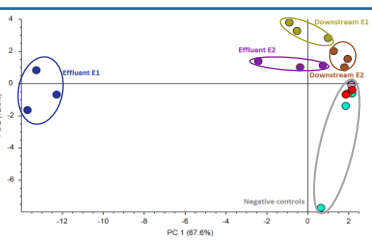


Fig.3 PCA plot of 31 signals selected from pull-down sample Effluent and Downstream

Non-target identification of protein ligands

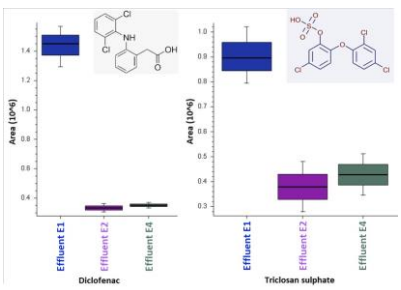


Fig.4 Box plot of 2 identified compounds from Effluent sample using LC-HRMS analysis

RESULTS

- Successfully **expressed** and **purified** functional **hTTR**
- Pull-down assay for hTTR establiished and optimized
- **Bioactivity after pull-down** of 2 field samples **confirmed**
- **Novel hTTR ligands discovered** e.g. diclofenac; triclosan sulphate; metoprolol; telmisartan; citalopram
- Contribution of individual ligands to the overall effect to be determined in mass balance calculations
- **Novel pull-down method is a great tool for identification of effect drivers in complex environmental samples.**

FURTHER PERSPECTIVES

- **Enhancement** of protein-ligand **binding selectivity** and ligand **recovery**
- Potential **combination** with **Effect directed analysis**
- **Expression of other proteins**
- **Application of pull-down assay for other environmental matrices.**

References

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