Center of Pharmacology and Toxicology
Institute of Pharmacology

Hannover Medical School

Development of tools for a better understanding of 3',5'-cUMP function in cells

Bastian Schirmer[1], Richard Kromke[1], Kristin Engel[1], Arslan Hussein[1], Heike Bähre[2], Annika Bosse[1], Tim Dolgner[1], Roland Seifert[1,2]

1 *Institute of Pharmacology, Hannover Medical School, Hannover, Germany*
2 *Research Core Unit Metabolomics, Hannover Medical School, Hannover, Germany*

Introduction

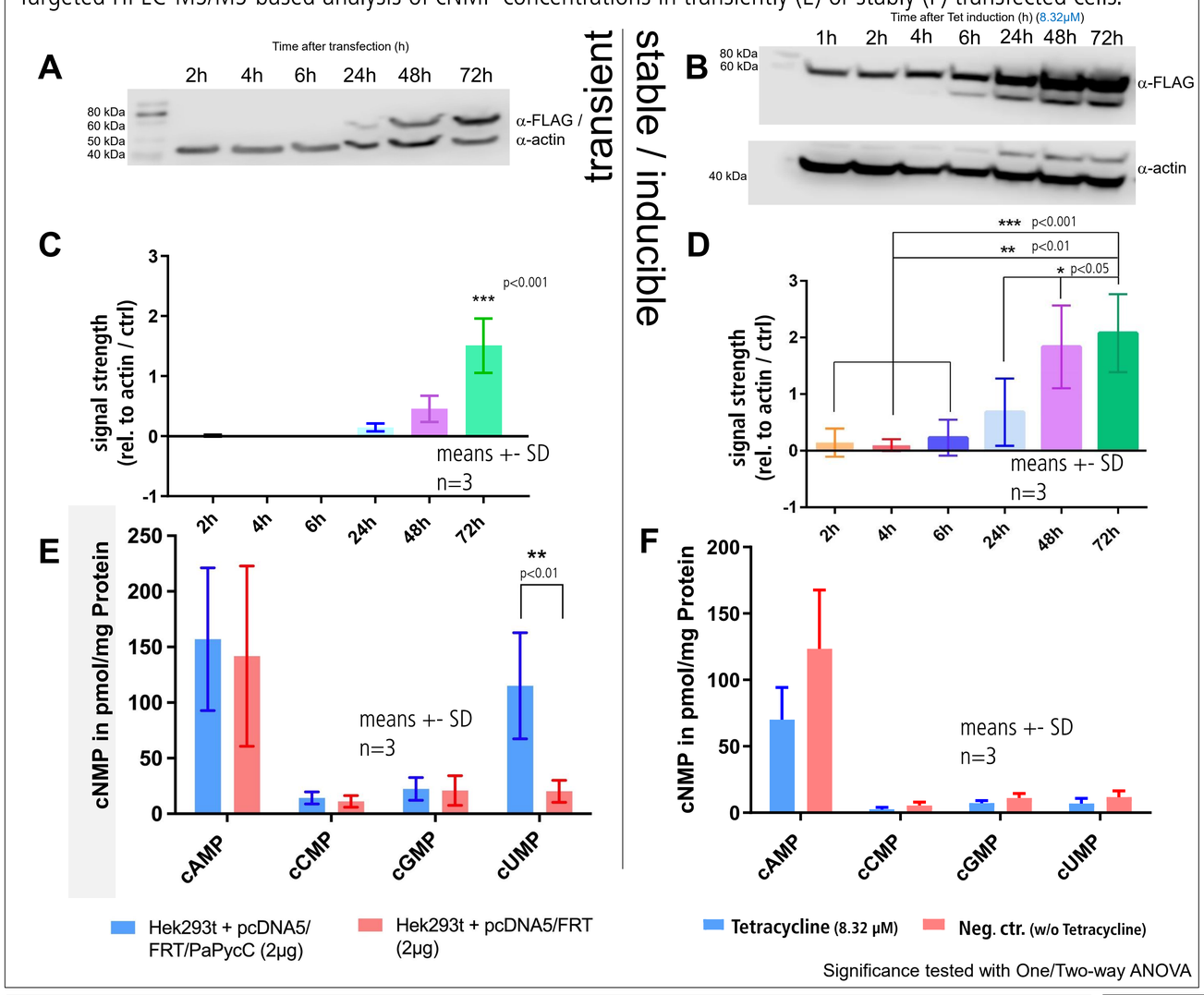
The presence of 3',5'-cyclic uridine monophosphate (cUMP) in cells has already been shown by sensitive and specific mass spectrometric methods. However, the generators and effectors of these nucleotide species and thus their biological functions are not sufficiently researched. A first indication for a dedicated function of cUMP in bacteria was provided by the discovery of the central role of cUMP in one of the bacterial anti-phage defense systems, which has been termed pyrimidine cyclase system for antiphage resistance (Pycsar). Our hypothesis is that the identified Pycsar proteins can be used as tools for further research on cellular effects of cUMP.

Methods

The Pycsar cUMP generator PaPycC of *Pseudomonas aeruginosa* was cloned into eukaryotic expression vectors allowing transient and inducible expression of either native or His-tagged protein. Enzymatic activity and substrate selectivity were tested using highly sensitive mass spectrometry (MS). The Pycsar cUMP effector PaPycTIR was cloned into a eukaryotic expression vector allowing expression of the protein within a FRET frame (mTurquoise2/mVenus). Starting from the core cyclic nucleotide binding domain, truncated forms of the enzyme were generated by targeted mutagenesis with the aim of yielding a biosensor for cUMP. FRET intensity was measured in cellular lysates using a microplate reader. AlphaFold3-based structure prediction was used to complement experimental data. Non-targeted MS-based metabolomics were used to uncover possible effectors of cUMP in eukaryotic cells.

Results

Fig. 1: Analysis of *Pycsar PaPycC* cUMP generator in the eukaryotic cell context. Western blot detection of PaPycC expression kinetics in 293 cells transiently (A) or stably (B) transfected. Beta-actin was used as reference protein for normalization in densitometric analysis of the kinetics in transiently (C) or stably (D) transfected cells. Targeted HPLC-MS/MS-based analysis of cNMP concentrations in transiently (E) or stably (F) transfected cells.



Significance tested with One/Two-way ANOVA

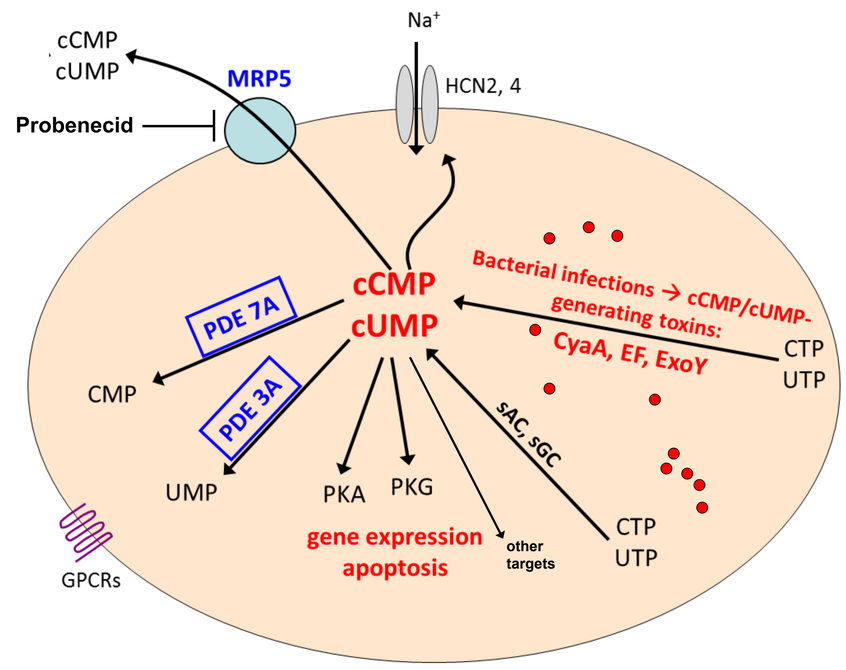
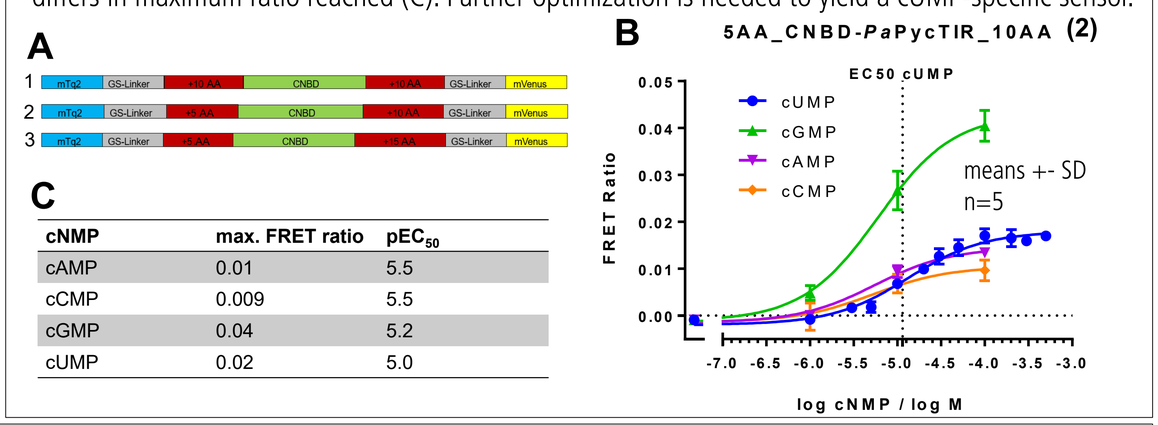


Fig. 2: Generation of a *Pycsar PaPycTIR*-based cUMP FRET sensor. (A) Examples of mutants generated based on a step-wise reconstitution of the PyPycTIR protein from the minimum cyclic nucleotide binding domain (CNBD). (B) For the FRET sensor 2, measurements of FRET ratios show a cNMP-dependent increase. The sensor responds to all analysed cNMPs with almost equal pEC_{50} but differs in maximum ratio reached (C). Further optimization is needed to yield a cUMP-specific sensor.



C

cNMP	max. FRET ratio	pEC_{50}
cAMP	0.01	5.5
cCMP	0.009	5.5
cGMP	0.04	5.2
cUMP	0.02	5.0

Fig. 3: Computer-aided structure prediction of FRET sensors.

(A-C) Structure prediction of PaPycTIR-based FRET sensors 1 (A), 2 (B), and 3 (C) with AlphaFold3. (D) Docking emulation of cUMP to FRET sensor 2 with Auto Dock Vina / PyMOL. The enlarged panel shows amino acids that are predicted to stabilize the binding of cUMP to the sensor.

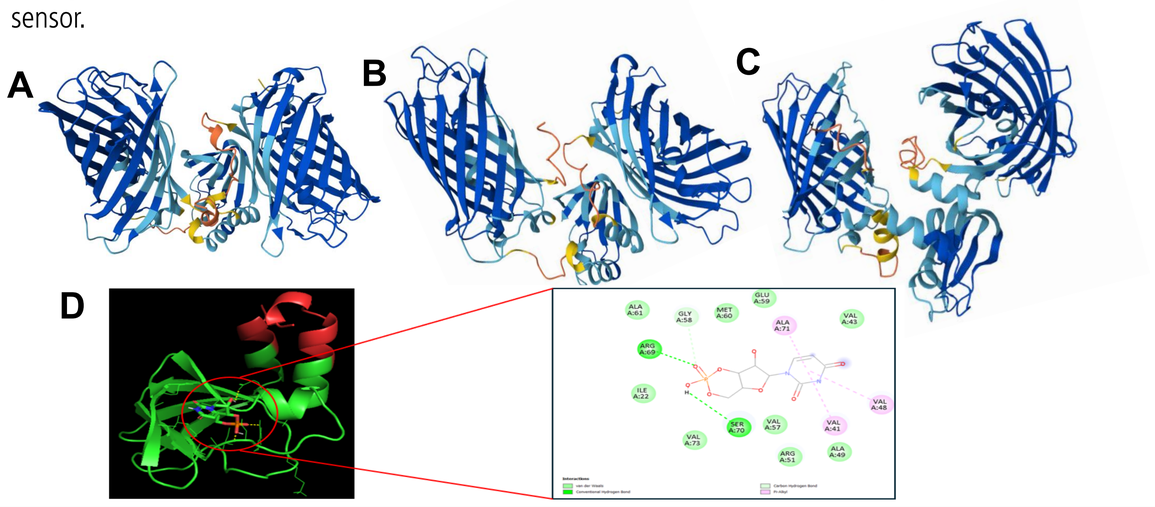
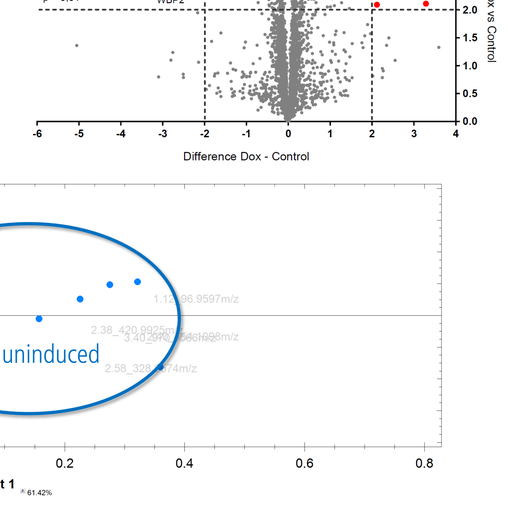


Fig. 4: Non-targeted mass-spectrometric analyses.

(A) MS-based (Vion™ IMS Q-Tof™, Waters) metabolomic comparison of tetracyclin treated 293 cells stably expressing tetracyclin-inducible ***PaPycC***. Data is reduced to show optimal variance without correlation (Principal Components Analysis), thus maximizing differences in metabolic markers. (B) Proof-of-principle pilot proteomics experiment with tetracyclin-treated 293 cells stably expressing tetracyclin-inducible ***EcPycC***, a cCMP generator.



Conclusion/Outlook

The Pycsar cUMP generator PaPycC and the corresponding effector PaPycTIR are promising candidates for further optimization to turn them into tools for selective analysis of cUMP effects in living eukaryotic cells. Exchanging the mTq2 fluorophore for a nanoluciferase module in an optimized biosensor will then possibly yield a useful real-time live cell sensor for cUMP.

Acknowledgements
Mass spectrometric measurements have been done by the research core unit metabolomics and proteomics of Hannover Medical School.

Metabolomics

DFG
INST 192/524-1 FUGG
Projekt-ID GGF 886

human brain only AI

ANR DFG Deutsche Forschungsgemeinschaft
toxi-cUMPsignal

MHH
Pharmacology OE 5320
Carl-Neuberg-Straße 1, 30625 Hannover, Germany
Schirmer.Bastian@mh-hannover.de, www.mhh.de/pharmakologie