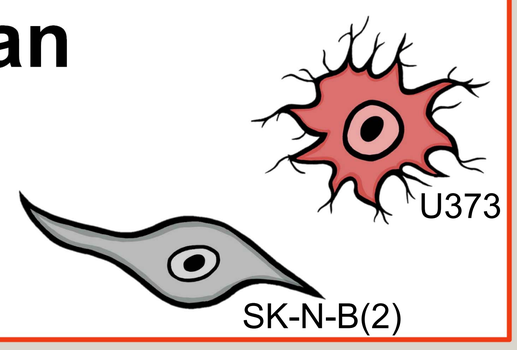
Institute of Pharmacology

Hannover Medical School

Anti-proliferative effect of cyclic nucleotides in human glioblastoma and neuroblastoma cell lines



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Introduction

Glioblastoma is the most aggressive and common malignant primary brain tumor of adulthood. In contrast, neuroblastoma is the most common malignant extracranial tumor of infancy, which occurs in the sympathetic nervous system. Therapy options are still limited for both (1, 2).

The canonical second messengers cAMP and cGMP regulate many intracellular processes like cell growth and differentiation. Further, the non-canonical second messengers cCMP and cUMP induce apoptosis in mouse lymphoma, human erythroleukemia, myelogenous and breast cancer cell lines (3).

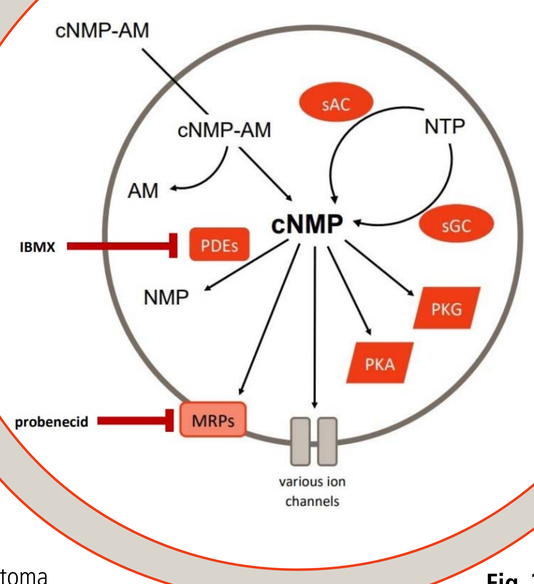
In this study we analyze the effect of cNMP-AMs in proliferation in the human glioblastoma U373, and neuroblastoma SK-N-B(2) cell line.

Methods

cNMP concentrations were measured by HPLC-MS/MS. To determine expression of MRP 4 and 5, PDE-isoforms, sAC and sGC-isoforms, qRT-PCR was performed.
To imitate the functions of cNMPs the membrane-permeable acetoxymethylester analogues (cNMP-AMs) were used, which release cNMP after intracellular hydrolysis.

The cell viability was analyzed by the alamarBlue assay. 2 x 10^4 cells in a volume of 100 µl were plated in a 96-well plate in triplicates. After 24 h, the cells were treated with cNMP-AMs for 24 h, 48 h, and 72 h. AlamarBlue (10 µl per well) was added 4h before measurement. To increase cNMP-concentration in the cell, export and degradation were inhibited by non-specific PDE-inhibitor (IBMX) and non specific MRP-inhibitor (probenecid).

Apoptosis were investigated by caspase 3 activation.



1. cNMP concentrations in U373 and SK-N-B(2) cells

	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)	cUMP (pmol/mg protein)	cCMP (pmol/mg protein)
U373	28.9 ± 3.1	3.1 ± 0.3	4.5 ± 0.7	1.9 ± 0.3
SK-N-B(2)	11.1 ± 2.2	1.3 ± 0.3	2.3 ± 0.4	0.9 ± 0.2

Fig. 1: cAMP, cGMP, cUMP and cCMP concentration in glioblastoma (U373) and neuroblastoma (SK-N-B(2)) cell lines. cNMP concentrations were measured by HPLC-MS/MS analysis. Means ± SD, n = 3 in triplicates.

All cNMPs were detectable in U373 and SK-N-B(2) cells. cAMP shows highest values in both cell lines, followed by cUMP, cGMP and cCMP.

2. Expression profile of MRPs, PDE-isoforms, sAC and sGC-isoforms in U373 and SK-N-B(2) cells

	MRP		PDE				NC		
	4	5	3A	3B	7A	9A	sAC	cGC *GUCY1A3*	sGC *GUCY1B3*
U373	7.9 ± 0.2	6.8 ± 0.5	15.8 ± 1.1	18.8 ± 0.1	12.7 ± 0.4	5.6 ± 0.3	15.5 ± 0.7	10.9 ± 0.2	7.8 ± 0.2
SK-N-B(2)	3.8 ± 0.8	1.7 ± 0.5	6.7 ± 0.7	5.6 ± 0.3	9.5 ± 0.3	4.5 ± 0.4	10.3 ± 0.4	0.1 ± 1.5	2.9 ± 1.2

Fig. 2: Expression of MRP 4 and 5, cCMP- or cUMP-degrading PDE-isoforms, sAC and sGC-isoforms. mRNA expression was analyzed by qRT-PCR. C_t values were normalized to ActB C_t values. Shown are means of ΔC_t values ± SD, n = 3 in duplicates. High expression $\Delta C_t < 5$ marked in blue; low expression $\Delta C_t > 10$ marked in red.

High expression of MRP 4 and 5 in SK-N-B(2) cells. PDE-isoforms are expressed low in U373 cells. sAC is expressed low in both cell lines, sGC-isoforms are expressed on a higher level.

3. Probenecid enhances the effect of cNMP-AMs after 72h, IBMX shows no further enhancement

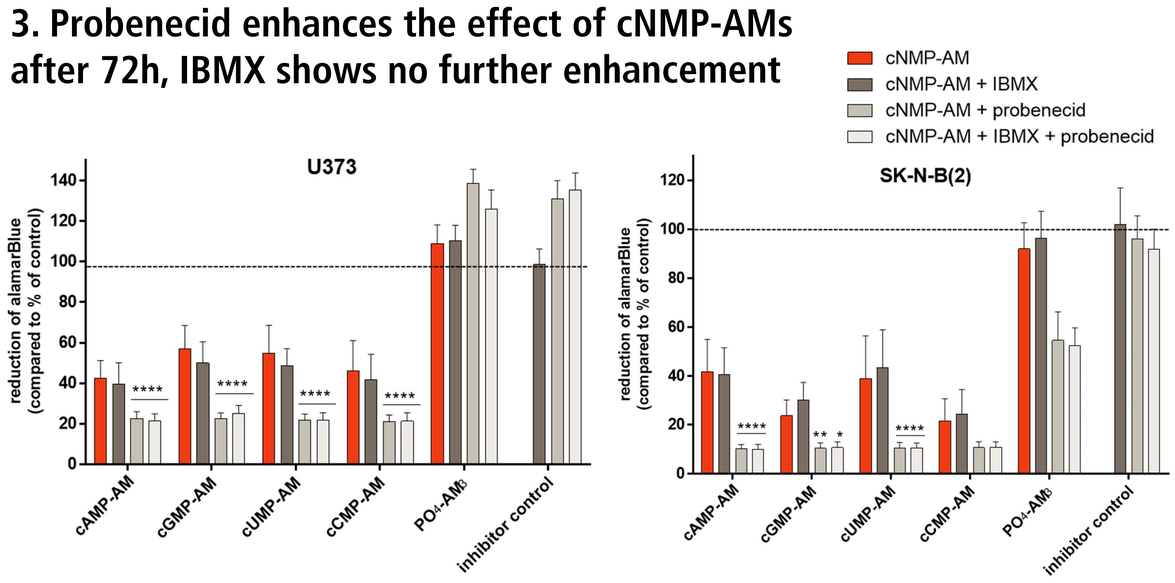


Fig. 3: AlamarBlue assay of cNMP-AM treated U373 and SK-N-B(2) cells. Cells were pre-incubated with 100 µM IBMX and/ or 500 µM probenecid. After that cells were incubated with 100 µM cNMP-AMs or 33 µM PO_4-AM_3. Means ± SD, n=5 in triplicates, two-way ANOVA with Tukey's multiple comparisons test compared to control. (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0{,}0001$).

The anti-proliferative effect was enhanced after pre-incubation with probenecid. IBMX alone/ in combination with probenecid shows no further enhancement. In U373 cells the effect is significant for all cNMP-AMs. In SK-N-B(2) cells there is a high significance for cAMP-AM and cUMP-AM.

4. Treatment with cNMP-AMs leads to activation of caspase 3

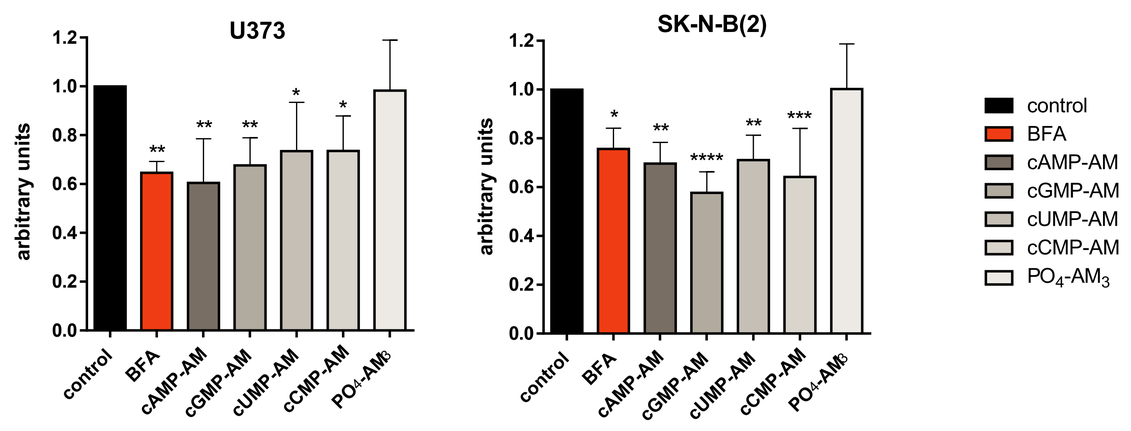


Fig. 4: Western blot analysis of caspase 3 protein expression in treated U373 and SK-N-B(2) cells. Cells were incubated with 100 µM cNMP-AMs, 33 µM PO_4-AM_3 or 5 µM BFA. Protein expression of full length caspase 3 and β-actin was determined by western blotting and quantified using image studio lite software. Protein bands were normalized to the expression of β-actin and control was calculated to 1. Shown are means ± SD, n = 5 – 7, one-way ANOVA with Dunnett's multiple comparisons test. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0{,}0001$).

Treatment with cNMP-AMs leads to reduction of caspase 3 full length in U373 and SK-N-BE(2) cells and induction of apoptosis. Caspase 3 is the major executioner caspase and the primary downstream mediator of apoptotic-associated proteolysis.

Summary and outlook

- **All cNMPs are detectable in U373 and SK-N-B(2) cells, with cAMP having the highest value (cAMP > cUMP > cGMP > cCMP).**
- **U373 and SK-N-B(2) cells show a different expression profile of genes that are important for the cNMP-metabolism.**
- **sGC-isoforms (*GUCY1A3, GUCY1B3*) are expressed higher than sAC in both cell lines.**
- **All cNMP-AMs showed a time and cell line specific anti-proliferative effect in U373 and SK-N-B(2) cells.**
- **Pre-incubation with probenecid increased the anti-proliferative effect. IBMX alone or in combination with probenecid has no further anti-proliferative effect.**
- **cNMP-AMs induced apoptosis in U373 and SK-N-B(2) cells.**

→ Combinations of cNMP-AMs and different concentrations of cNMP-AMs should be studied.
→ PDE-isoform specific inhibitors should be investigated.
→ Primary cells from patients should be analyzed.

References

[1] De Vleeschouwer S., *et al*, Glioblastoma (2017)
[2] Mahapatra S., *et al*, Neuroblastoma (2023)
[3] Wolter S., *et al*, cCMP and cUMP in Apoptosis: Concepts and Methods (2017)

Acknowledgement

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