Institute of Pharmacology

Hannover Medical School

MRP4/5-inhibitor probenecid enhances anti proliferative effects of cNMPs in human gynecological cancer cell lines

Sabine Wolter, Lea Giller, Roland Seifert

Institute of Pharmacology, Hannover Medical School, Hannover, Germany

Introduction

Gynecological tumors, including tumors of the female breast and genital organs, pose a significant health burden worldwide, and the development of new therapies is necessary.

The canonical second messengers cAMP and cGMP regulate many intracellular processes like cell growth and differentiation. Furthermore, the non-canonical second messengers cCMP and cUMP induce apoptosis in mouse lymphoma, human erythroleukemia, myelogenous and breast cancer cell lines (1).

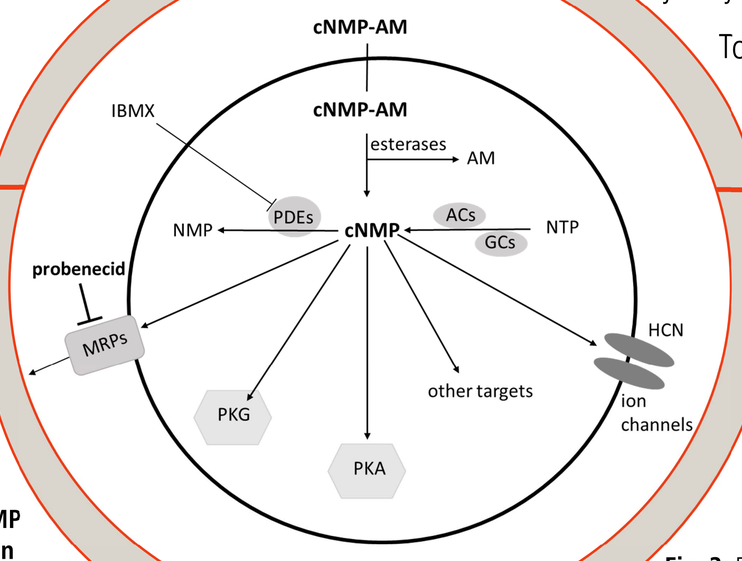
Multidrug resistance transporters (MRPs) 4 and 5 are expressed in many tumor cells and play an important role in the export of cNMPs, thereby terminating their actions.
In this study, we analyze the effect of cNMP-AMs on proliferation in various human carcinoma cell lines.

Methods

We used the breast cancer cell lines MCF-7 and ZR-75-1, and the TNBC cell line MDA-MB-231 (MDA). In addition, the ovarian cancer cell lines CAOV-3, PA-1, SK-OV-3, and SW626, as well as the endometrial cancer cell line (HEC-1-B), were included.
cNMP concentrations were measured by HPLC-MS/MS. To determine the expression of MRP4 and MRP5, qRT-PCR and western blot analyses were performed.

To imitate the functions of cNMPs the membrane-permeable acetoxymethylester analogues (cNMP-AMs) were used, which release cNMP after intracellular hydrolysis. Cell viability was analyzed by using the alamarBlue assay.

To increase cNMP-concentration in the cell, export and degradation were inhibited by non-specific PDE-inhibitor (IBMX) and non-specific MRP-inhibitor (probenecid).



1. cNMP concentrations in carcinoma cell lines

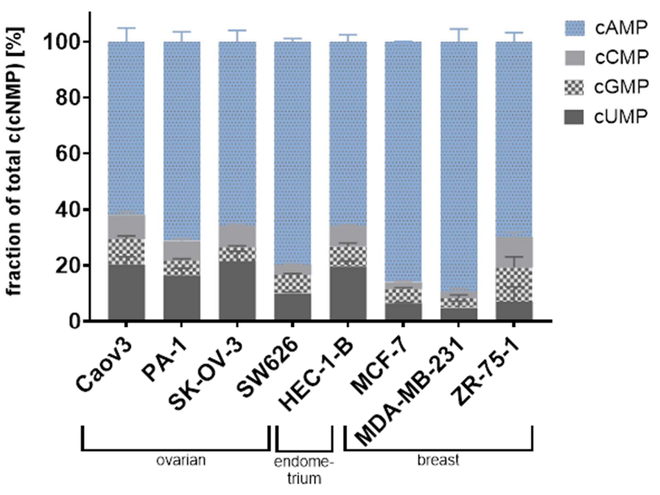


Fig. 1: Relative cNMP concentration
cAMP, cGMP, cUMP and cCMP concentrations were measured by HPLC-MS/MS analysis. Means ± SD, n = 3 in triplicates.

All cNMPs were detectable and showed a cell type specific pattern.

2. Expression profile of MRPs and PDE-isoforms

	MCF-7	MDA	ZR-75-1	HEC-1-B	CAOV-3	PA-1	SK-OV-3	SW626
MRP4	8.0 ± 0.5	5.7 ± 0.6	8.4 ± 0.2	**3.0± 1.5**	7.3 ± 1.8	7.0 ± 1.0	**3.5 ± 1.4**	6.4 ± 0.7
MRP5	**4.8 ± 0.1**	5.9 ± 0.5	6.8 ± 0.1	**5.0 ± 0.9**	**4.8 ± 1.5**	6.1 ± 0.7	6.1 ± 0.7	5.9 ± 1.0
PDE3A	**17.3 ± 0.1**	**13.8 ± 1.0**	**13.5 ± 0.5**	**12.7 ± 1.5**	8.0 ± 1.8	7.6 ± 2.2	7.6 ± 2.2	**11.5 ± 0.7**
PDE3B	8.5 ± 0.6	9.4 ± 0.7	5.4 ± 1.0	7.4 ± 1.4	**13.2 ± 1.4**	6.6 ± 0.9	6.6 ± 0.9	**10.1 ± 0.7**
PDE7A	9.0 ± 0.8	9.7 ±0.3	7.7 ± 1.1	**11.6 ± 2.1**	8.9 ± 1.5	**10.1 ± 1.5**	**10.1 ± 1.5**	**10.0 ± 1.0**
PDE9A	6.5 ± 1.7	8.1 ± 0.2	**4.3 ± 0.9**	**2.7 ± 1.2**	**4.2 ± 1.9**	**5.0 ± 1.9**	**5.0 ± 1.9**	**4.6 ± 0.5**

Fig. 2: Expression of MRP 4 and 5, and cCMP- or cUMP-degrading PDE-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 $\Delta C_t \leq 5$ marked in red; low expression $\Delta C_t \geq 10$ marked in blue.

High expression of MRP4 and/or MRP5 were detected in MCF-7, HEC-1-B, SK-OV-3 and CAOV-3 cells. PDE7A (cCMP-degrading isoform) is expressed at low levels HEC-1-B, PA-1, SK-OV-3 and SW626 cells. cUMP-degrading isoform (9A) are expressed at high level in ZR-75-1, HEC-1-B, CAOV-3, PA-1, SK-OV-3, and SW626 cells.

3. Probenecid enhances the effect of cNMP-AMs after 72h in ZR-57-1 and SW626 cells, IBMX shows no further enhancement

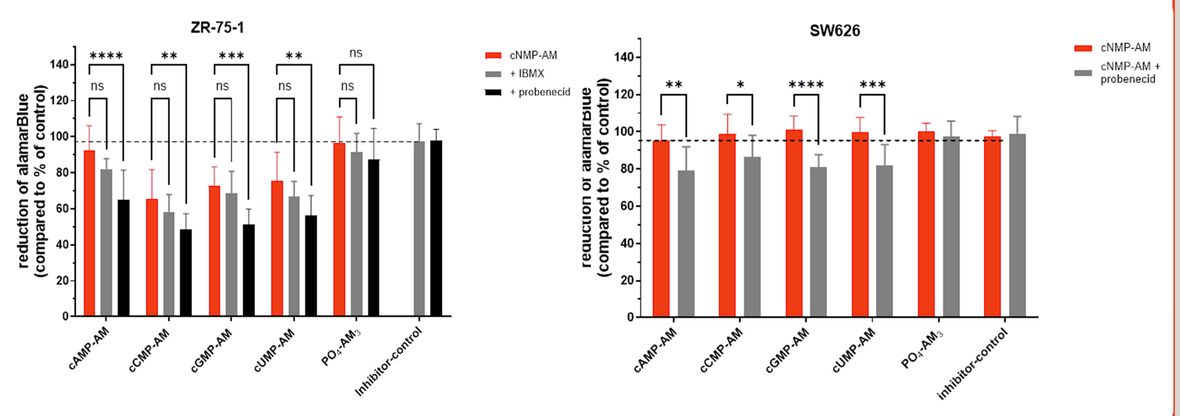


Fig. 3: AlamarBlue assay of cNMP-AM treated ZR-75-1 and SW626 cells. Cells were pre-incubated with 100 µM IBMX and/or 500 µM probenecid. After that cells were incubated with 100 µM cNMP-AMs or 33 µM PO_4-AM_3. Means ± SD, n=5 in triplicates, two-way ANOVA with Tukey's multiple comparisons test compared to control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

The anti proliferative effect was enhanced after pre-incubation with probenecid in both cell lines. IBMX alone as well as in combination with probenecid shows no further enhancement. The effect is significant for all cNMP-AMs.

4. MRP4 and MRP5 protein expression in carcinoma cell lines

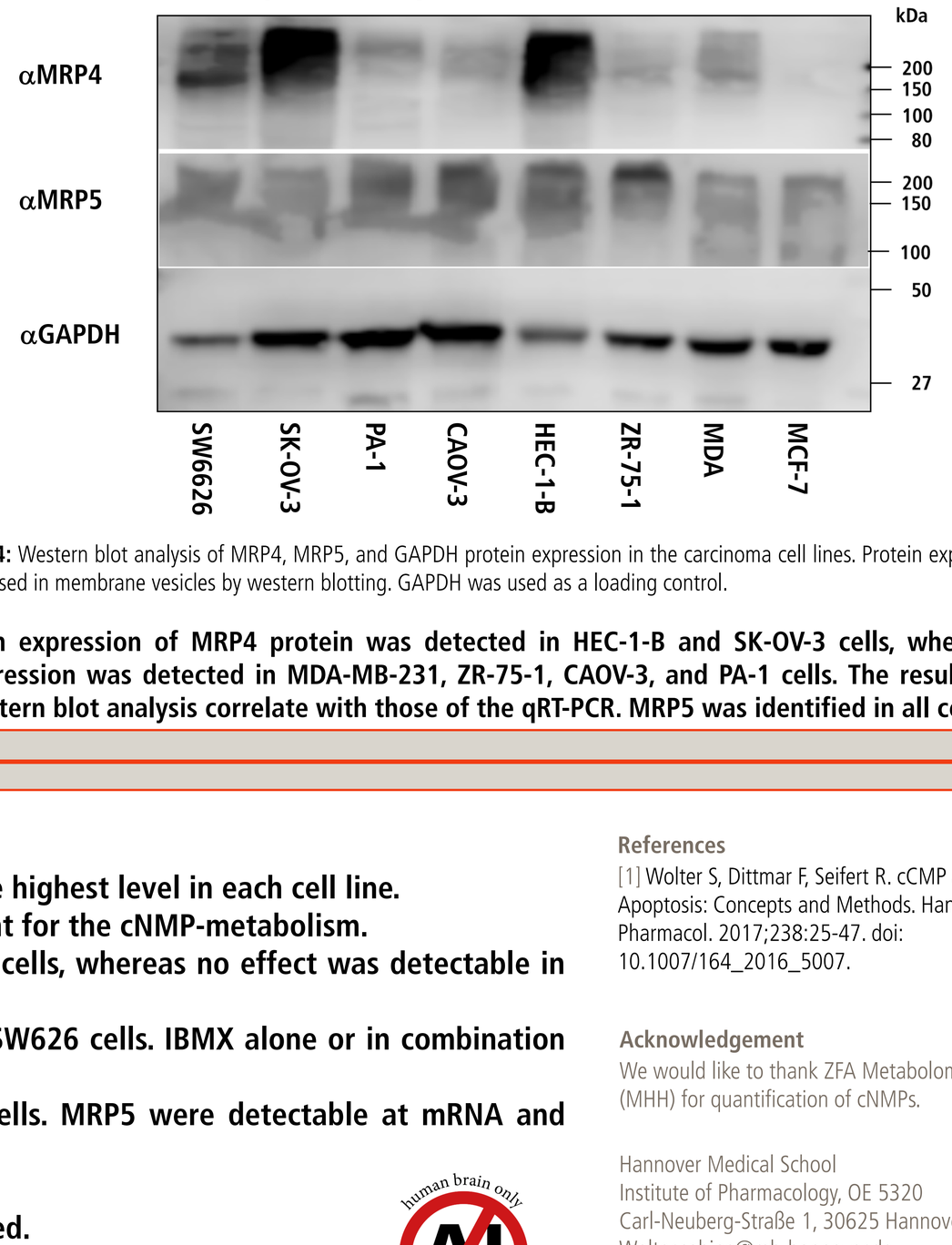


Fig. 4: Western blot analysis of MRP4, MRP5, and GAPDH protein expression in the carcinoma cell lines. Protein expression was analysed in membrane vesicles by western blotting. GAPDH was used as a loading control.

High expression of MRP4 protein was detected in HEC-1-B and SK-OV-3 cells, whereas low expression was detected in MDA-MB-231, ZR-75-1, CAOV-3, and PA-1 cells. The results of the western blot analysis correlate with those of the qRT-PCR. MRP5 was identified in all cell lines.

Summary and outlook

- **All cNMPs were detectable in every carcinoma cell line examined, with cAMP having the highest level in each cell line.**
- **Each carcinoma cell line showed a distinct expression profile of genes that are important for the cNMP-metabolism.**
- **All cNMP-AMs showed a time and cell line specific anti-proliferative effect in ZR-75-1 cells, whereas no effect was detectable in SW626 cells.**
- **Pre-incubation with probenecid increased the anti proliferative effect in ZR-75-1 and SW626 cells. IBMX alone or in combination with probenecid had no further anti proliferative effect.**
- **MRP4 were expressed at high level at mRNA and protein in HEC-1-B and SK-OV-3 cells. MRP5 were detectable at mRNA and protein level in all carcinoma cell lines.**

→ Combinations of cNMP-AMs and different concentrations of cNMP-AMs should be studied.
→ PDE-isoform-specific inhibitors should be investigated.
→ MRP-specific inhibitors should be studied.

References

[1] Wolter S, Dittmar F, Seifert R. cCMP and cUMP in Apoptosis: Concepts and Methods. Handb Exp Pharmacol. 2017;238:25-47. doi: 10.1007/164_2016_5007.

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Hannover Medical School
Institute of Pharmacology, OE 5320
Carl-Neuberg-Straße 1, 30625 Hannover
Wolter.sabine@mh-hannover.de
www.mhh.de/pharmakologie