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Membrane-Permeant, Bioactivatable Analogues of cGMP as Inducers of Cell Death in IPC-81 Leukemia Cells

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Abstract—We report an improved single-step synthesis to generate the membrane-permeant acetoxymethyl esters (AM-esters) of cGMP and three cGMP-analogues. These bioactivatable compounds were found to induce cell death in rat IPC-81 cells, a model system for acute myelocytic leukemia, in micromolar doses, while the corresponding non-modified cGMP-analogues were inactive.

Guanosine 3′,5′-cyclic monophosphate (cGMP) is a key second messenger, mediating physiological effects in most mammalian cells. It acts through the regulation of several distinct receptor proteins, including cyclic nucleotide-gated ion channels, cGMP-regulated phosphodiesterases, and cGMP-dependent protein kinases.[1] Mainly due to its polar ionic structure, cGMP is not able to penetrate intact cellular membranes, a severe problem for experiments with living cells. To overcome this problem cGMP-analogues with hydrophobic substituents have been synthesized and widely used to elucidate the functional role of the cGMP signal cascade in biological systems, like relaxation of smooth muscle, Cl^- secretion from intestinal epithelium, and inhibition of platelet aggregation.[2–4] However, the biological potency of cGMP is often decreased by substituents. Poor membrane permeability still remains a major limitation of the usefulness of 8-bromo-cGMP and similar derivatives. Therefore, millimolar concentrations of the compounds in the extracellular medium are often necessary to produce cellular effects.[3–5]

Schultz et al. successfully designed a new generation of bioactivatable cyclic nucleotides with strongly enhanced membrane permeability, by masking the negative phosphate with an acetoxymethyl ester (AM-ester) group, including N^6,$O^{2'}$-dibutyryl-cAMP/AM, cGMP/AM, and N^2,$O^{2'}$-dibutyryl-cGMP/AM.[6,7] More recently, this synthetic method was extended to 8-substituted derivatives of cAMP by Kruppa et al., employing an approach which allowed direct alkylation of the unprotected sodium salts.[8] We adapted and modified the latter method to convert cGMP and three cGMP-analogues into their corresponding AM-esters.

The acetoxymethyl esters (**1**–**4**) were applied extracellularly to IPC-81 cells, a cell line considered as a rat model for human acute myelocytic leukemia, and their potency to induce cell death was compared to cGMP and the non-alkylated cGMP-derivatives, respectively.

Results and Discussion

Synthesis

Initial AM-ester preparations were carried out in dimethylformamide (DMF) as an aprotic, dipolar solvent, following the procedure of Kruppa et al. Some cGMP-analogues showed insufficient solubility in DMF and the formation of several products after 1 h reaction time, as was verified by high-performance liquid chromatography (HPLC). By-product formation appeared to be the main limitation of this procedure. Solubility problems were prevented by a 1:1 (v:v) mixture of DMF with dimethylsulfoxide (DMSO). Successful regioselective alkylation of the cyclic phosphate by addition of five equivalents of acetoxymethyl bromide (AM-Br) and

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diisopropylethylamine (DIEA) was now possible within 5–15 min. The progress of AM-ester formation was controlled in minute intervals by HPLC, and the reactions were stopped by shock freezing before significant amounts of side products where detectable. With this new single-step procedure the cyclic nucleotide AM-esters cGMP/AM (**1**), 8-Br-cGMP/AM (**2**), 8-*p*CPT-cGMP/AM (**3**), and 1-CH_3-cGMP/AM (**4**) (Fig. 1) were obtained in overall yields of 17% (**1**) to 22% (**4**) in purities higher than 98% after isolation by semi-preparative HPLC.

Cell viability studies

Potent cAMP-analogues or other stimuli that elevate intracellular cAMP levels induce apoptotic cell death in IPC-81 cells, by activation of the cAMP-dependent protein kinase type I.[9] Given the fact that the participation of cGMP as a second messenger in the modulation of apoptosis is of growing interest,[10,11] we tested cGMP/AM (**1**), 8-Br-cGMP/AM (**2**), cGMP, and 8-Br-cGMP as initiators of cell death on IPC-81 cells. Figure 2A and B illustrates the cell viabilities monitored by MTT-assays[12] 24 h after extracellular application of the compounds. cGMP/AM and 8-Br-cGMP/AM (**2**) induced cell death with EC_{50}-values in the mid micromolar range (40 and 80 μM, respectively), while cGMP and 8-Br-cGMP were completely inactive even in millimolar doses. Preliminary experiments suggested that 8-*p*CPT-cGMP/AM (**3**) and 1-CH_3-cGMP/AM (**4**) were active in similar or slightly higher concentrations, respectively (data not shown). We conclude that only the AM-esters **1** and **2** but not the non-modified charged compounds cGMP and 8-Br-cGMP are of sufficient hydrophobicity to penetrate the cell membranes. After cleavage of the AM-ester group by endogenous esterases the two resulting cyclic nucleotides, cGMP and 8-Br-cGMP caused cell death in this leukemia cell line.

Our results provide first hints for a role of cGMP and cGMP receptor proteins as death-signals in IPC-81 cells. Further work will be needed to explore the molecular basis of cGMP/AM-analogue action and the nature of induced cell death (apoptotic or necrotic) in this cell line.

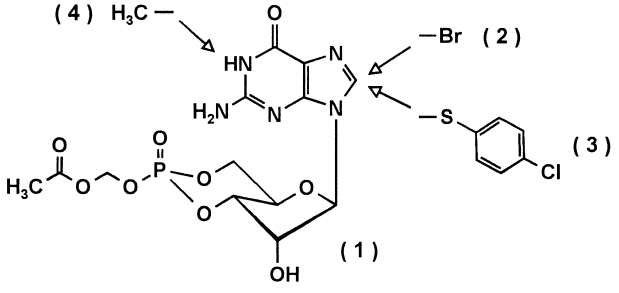

Figure 1. Chemical structures of cGMP/AM (**1**), 8-bromo-cGMP/AM (8-Br-cGMP/AM) (**2**), 8-*p*-chlorophenylthio-cGMP/AM (8-*p*-CPT-cGMP/AM) (**3**), and 1-methyl-cGMP/AM (1-CH_3-cGMP/AM) (**4**); (only the R_P-diastereomers are shown).

Materials and Methods

DMF, DMSO, and DIEA were purchased in the highest purity available and stored over activated molecular sieves (3 Å). AM-Br was from Aldrich, cGMP, 8-*p*CPT-cGMP, and 8-Br-cGMP were from BIOLOG Life Science Institute, Bremen. 1-CH_3-cGMP was synthesized as described before.[13] NMR spectra were recorded on a Bruker WH-360 spectrometer in DMSO-d_6, mass spectra were performed on a Finnigan MAT Model 8222, with FAB ionisation and glycerol as matrix.

General synthetic procedure

One hundred micromoles of cyclic nucleotide was dissolved in 1 mL dry solvent (DMF:DMSO, 1:1, v:v) under argon protection. Five hundred micromoles DIEA and 500 μmol AM-Br were added, the reaction mixture was kept for 5–15 min at room temperature. The reaction was stopped by shock-freezing in liquid nitrogen. All volatile components were removed in vacuum. Purification of the AM-esters was performed on a semi-preparative HPLC column (Merck LiChrosorb® RP 18, 10 μm, 250×10 mm). The structure of each AM-ester

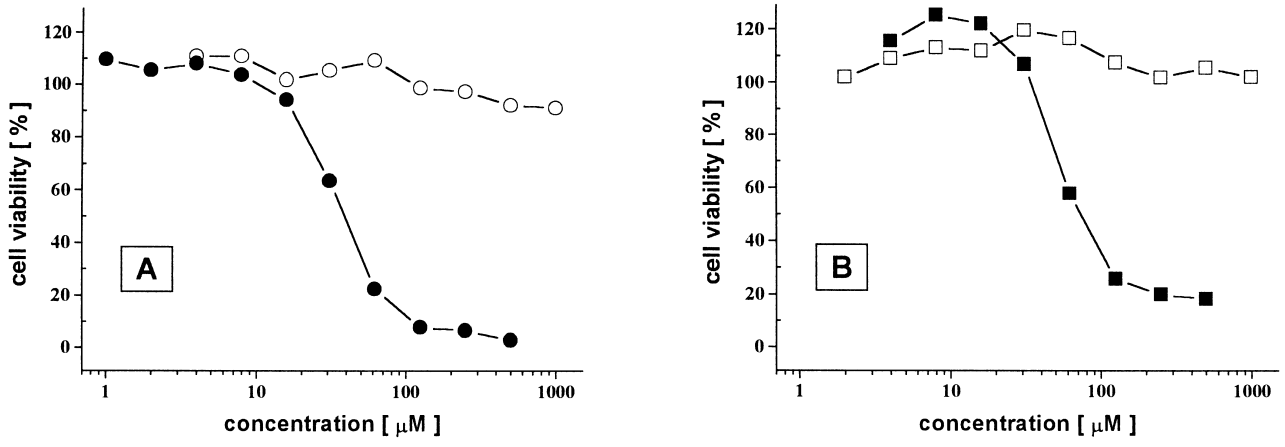

Figure 2. Dose–response curve profiles of IPC-81 cell viability: data were aquired 24 h after extracellular addition of the compounds. (**A**) cGMP [–○–] and cGMP/AM (**1**) [–●–], (**B**) 8-Br-cGMP [–□–] and 8-Br-cGMP/AM (**2**) [–■–].

(**1**–**4**) was confirmed by NMR and MS spectroscopy. In all cases both diastereomers of the acetoxymethyl esters (R_p and S_p) were produced in similar amounts.

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