Apoptotic and anti-proliferative effect of guanosine and guanosine derivatives in HuT-78 T lymphoma cells

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Abstract
The effects of 100 μM of 3′,5′-cGMP, cAMP, cCMP, and cUMP as well as of the corresponding membrane-permeant acetoxymethyl esters on anti-CD3-antibody (OKT3)-induced IL-2 production of HuT-78 cutaneous T cell lymphoma (Sézary lymphoma) cells were analyzed. Only 3′,5′-cGMP significantly reduced IL-2 production. Flow cytometric analysis of apoptotic (propidium iodide/annexin V staining) and anti-proliferative (CFSE staining) effects revealed that 3′,5′-cGMP concentrations >50 μM strongly inhibited proliferation and promoted apoptosis of HuT-78 cells (cultured in the presence of αCD3 antibody). Similar effects were observed for the positional isomer 2′,3′-cGMP and for 2′-GMP, 3′-GMP, 5′-GMP, and guanosine. By contrast, guanosine and guanosine-derived nucleotides had no cytotoxic effect on peripheral blood mononuclear cells (PBMCs) or acute lymphocytic leukemia (ALL) xenograft cells. The anti-proliferative and apoptotic effects of guanosine and guanosine-derived compounds on HuT-78 cells were completely eliminated by the nucleoside transport inhibitor NBMPR (S-(4-Nitrobenzyl)-6-thioinosine). By contrast, the ecto-phosphodiesterase inhibitor DPSPX (1,3-dipropyl-8-sulfophenylxanthine) and the CD73 ecto-5′-nucleotidase inhibitor AMP-CP (adenosine 5′-(α,β-methylene)diphosphate) were not protective. We hypothesize that HuT-78 cells metabolize guanosine-derived nucleotides to guanosine by yet unknown mechanisms. Guanosine then enters the cells by an NBMPR-sensitive nucleoside transporter and exerts cytotoxic effects. This transporter may be ENT1 because NBMPR counteracted guanosine cytotoxicity in HuT-78 cells with nanomolar efficacy (IC₅₀ of 25–30 nM). Future studies should further clarify the mechanism of the observed effects and address the question, whether guanosine or guanosine-derived nucleotides may serve as adjuvants in the therapy of cancers that express appropriate nucleoside transporters and are sensitive to established nucleoside-derived cytostatic drugs.

Keywords Nucleoside transporters · Guanosine · Apoptosis · Proliferation · Leukemia · T-cells

Abbreviations
ALL Acute lymphocytic leukemia
AMP-CP Adenosine-5′-(α,β-methylene)diphosphate, a CD73 ectonucleotidase inhibitor
2′,3′- or 3′,5′-cGMP Adenosine 2′,3′- or 3′,5′-cyclic monophosphate
3′,5′-cAMP Annexin V-APC Allophycocyanin-labeled annexin V
BSA Bovine serum albumin
cCMP Cytidine 3′-cyclic monophosphate
CEM-ARA-C/8C Arabinosylcytosine-resistant childhood T acute lymphoblastic leukemia cell line
CFSE 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
2′,3′- or 3′,5′-cGMP Guanosine 2′,3′- or 3′,5′-cyclic monophosphate
cIMP Inosine 3′,5′-cyclic monophosphate
cNMP Cyclic nucleoside monophosphate
cNMP-AM Cyclic nucleoside 3′,5′ monophosphate acetoxymethyl ester
cUMP Uridine 3′,5′-cyclic monophosphate

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Introduction

Cyclic nucleotides (cNMPs), specifically cAMP and cGMP, are well-established second messengers. Although involvement in intracellular signaling processes is considered the main function of these cNMPs, an increasing body of scientific literature reports on first messenger effects of extracellular cAMP and cGMP. A “cAMP-adenosine pathway” has been repeatedly demonstrated for various cell types (Godinho et al. 2015; Jackson and Raghvendn 2004). 3′,5′-cAMP is exported into the extracellular space, followed by enzymatic degradation to adenosine, which in turn activates G protein-coupled receptors (Godinho et al. 2015, Jackson and Raghvendn 2004). A similar pathway seems to exist for 2′,3′-cAMP (Verrier et al. 2011) and for 2′,3′-cGMP (Jackson et al. 2019). In addition, several reports suggest that extracellular degradation products of 3′,5′-cGMP exert biological effects in the brain (Albrecht et al. 2013; Saute et al. 2006; Soares et al. 2004). Moreover, the pyrimidine nucleoside uridine activates adenosine receptors (Yilmaz et al. 2008). Thus, the non-canonical cyclic nucleotide cUMP, which is currently discussed as a potential second messenger (Seifert et al. 2015; Berrisch et al. 2017; Ostermeyer et al. 2018; Scharrenbroich et al. 2019), may as well be exported and degraded to biologically active products. Preliminary results suggest that first- and second messenger effects of cyclic nucleotides are highly dependent on the investigated cell type (Schneider et al. 2015).

Here we report on the effects of extra- and intracellular cNMPs and their extracellular degradation products on HuT-78 Sézary lymphoma cells in three different functional readouts, namely T cell receptor (TCR)-mediated IL-2 production, apoptosis, and proliferation. Unmodified cNMPs were used to address extracellular effects. Membrane-permeant 3′,5′-cNMP acetoxymethyl esters (cNMP-AMs) that release the unmodified 3′,5′-cNMPs after cleavage by intracellular esterases were used to investigate intracellular actions. IL-2 production of HuT-78 T cell lymphoma cells (induced with αCD3 antibody (OKT3) in the absence of αCD28) was significantly inhibited by 3′,5′-cGMP, while cGMP-AM was ineffective. Experiments addressing proliferation and apoptosis of HuT-78 cells (cultured in the presence of αCD3) revealed anti-proliferative and pro-apoptotic effects, not only of 3′,5′-cGMP but also of 2′,3′-cGMP, 2′-GMP, 3′-GMP, 5′-GMP, and guanosine. The effects of these guanosine-derived compounds depended on the activity of NBMPR (S-(4-Nitrobenzyl)-6-thioinosine)-sensitive nucleoside transporters. By contrast, substances not related to guanosine were ineffective. Our observations may be explained by the hypothesis that HuT-78 cells metabolize 3′,5′-cGMP, 2′,3′-cGMP, 2′-GMP, 3′-GMP, and 5′-GMP to guanosine, which enters the cells by an NBMPR-sensitive nucleoside transporter and exerts cytotoxic effects. Further studies are needed to investigate whether guanosine or guanosine-derived nucleotides could be used as adjuvants in the chemotherapy of lymphomas expressing appropriate nucleoside transporters.

Materials and methods

Buffers, reagents, and cell culture media

CFSE (5(6)-carboxyfluorescein diacetate N-succinimidyl ester; Cat.# 21888) and propidium iodide solution (Cat.# P4864) was provided by Sigma Aldrich (Taufkirchen, Germany). Annexin-V-APC (Cat.# AnxA100) was purchased from MabTag (Frisoythe, Germany). Mouse monoclonal (OKT3) anti-CD3 antibody (Cat.# SAB4700041) was from Sigma Aldrich (Taufkirchen, Germany). Pacific blue-labeled anti-human CD3 (Cat.# 300417) and propidium iodide solution (Cat.# P4864) was obtained from Biolegend (London, UK). Anti-CD28 antibody (Cat.# SAB4700135–100 μg was purchased from Sigma Aldrich (Taufkirchen, Germany). For detection of IL-2, either the Duoset ELISA (Cat.# DY202) from R&D Systems (Minneapolis, MN, USA) was used or the ELISA MAX Standard Set for human IL-2 (Cat.# 431801) from Biolegend (London, UK).
DPSPX (1,3-dipropyl-8-p-sulfophenylxanthine; Cat.# A022) and NBMPR (S-(4-nitrobenzyl)-6-thioinosine; Cat.# N2255) were obtained from Sigma Aldrich (Taufkirchen, Germany). 2',3'-cGMP (Cat# G025–50), 3',5'-cGMP (Cat# G001–100), 2'-GMP (Cat# G022–10), 3'-GMP (Cat# G021–10), and AMP-CP (adenosine-5′-(α,β-methylene)diphosphate, sodium salt; Cat.# A070) were purchased from Biolog (Bremen, Germany). 5'-GMP disodium salt (Cat.# G8377) and guanosine (Cat. # G6752) were provided by Sigma Aldrich (Taufkirchen, Germany).

RPMI 1640 medium (Cat.# F7524) and fetal calf serum (Cat.# R8758) were purchased from Sigma Aldrich (Taufkirchen, Germany). L-Glutamine with penicillin/streptomycin (Gibco # 10378–016), MEM non-essential amino acids (100 x) and glutamine, sodium pyruvate, sodium salt; and streptomycin. Moreover, the medium was supplemented with 10% (v/v) of fetal bovine serum and 2 mM of L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 4 μg/ml anti-CD28 antibody.

**Cell culture**

HuT-78 cutaneous T lymphoma cells (Sézary lymphoma) were obtained from LGC Standard GmbH (Wesel, Germany) and maintained in RPMI 1640 medium supplemented with 10% (v/v) of fetal bovine serum and 2 mM of L-glutamine as well as 100 U/ml penicillin and 0.1 mg/ml streptomycin. Moreover, the medium was supplemented with 1% (v/v) of MEM non-essential amino acids (100 x) and 1 mM of sodium pyruvate (addition of 100 mM stock solution). The cells were cultured at 37 °C in the presence of 5% of CO2. Medium was renewed three times a week by diluting the 1 mM of sodium pyruvate (addition of 100 mM stock solution). Medium was renewed three times a week by diluting the 1 mM of sodium pyruvate (addition of 100 mM stock solution). Medium was renewed three times a week by diluting the 1 mM of sodium pyruvate (addition of 100 mM stock solution). Medium was renewed three times a week by diluting the 1 mM of sodium pyruvate (addition of 100 mM stock solution). Medium was renewed three times a week by diluting the 1 mM of sodium pyruvate (addition of 100 mM stock solution).

**IL-2 ELISA**

96-well plates were coated for 24 h with 50 μl of PBS containing 2 μg/ml of αCD3 antibody (OKT3). On the next day, the plates were washed twice with 1× PBS and then loaded with 50 μl of cell suspension/well (100,000 HuT-78 cells or 1.75 × 105 PBMCs per well) plus 50 μl media with the corresponding stimuli. After 24 h of incubation, the plate was centrifuged for 10 min at 500×g. After that, supernatant was removed and used for the ELISA experiment. ELISAs were performed according to the manufacturer’s instructions (Duoset, Cat. # DY 202, from R&D Systems or ELISA MAX Standard Set, Cat. # 431801, from Biolegend).

**Apoptosis assay with HuT-78 cells, ALL-cells, and PBMCs**

HuT-78 cells (1 × 10^5 cells/ml) were treated for 72 h (37 °C, 5% CO2) in αCD3-antibody-coated 24-well plates with cNMPs, NMPs, or nucleosides in the presence or absence of inhibitors (AMP-CP, DPSPX, NBMPR). To counteract potential degradation in case of incubation with AMP-CP or DPSPX, 100 μM of these inhibitors were not only added at the beginning of the incubation time but also after 24 h and 48 h. Thus, after 72 h, the samples contained up to 300 μM of AMP-CP or DPSPX. On the day of the measurement, the cells were centrifuged (300×g, 4 min, ambient temperature), suspended in 100 μl of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) and then incubated with annexin-V-APC for 30 min in the dark at ambient temperature. After that, each sample was diluted by addition of 200 μl of binding buffer. Propidium iodide (PI) solution (final concentration 625 ng/ml) was added immediately prior to flow cytometric quantitation of apoptosis, which was performed using a MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany). Apoptosis was analyzed by generating an annexin-APC/PI dot plot (APC/PI), which allows the discrimination of the following populations: lower left (LL) quadrant (annexin-V-APC- and PI-negative): viable cells; lower right (LR) quadrant (annexin-V-APC-positive, PI-negative): early apoptosis; upper right (UR) quadrant (positive for both annexin-V-APC and PI): late apoptosis and upper left (UL) quadrant (positive for PI, negative for annexin-V-APC: necrosis. The data were analyzed with the MACSQuantify software. The degree of apoptosis was defined as percentage of total cells in the LR and UR quadrant (% apoptotic cells).

In case of ALL cells, MSC feeder cells were seeded on Fridays on a 24-well plate (pre-coated for 24 h with 2 μg/ml of α-CD3 antibody) at a density of 1 × 10^4 cells/ml (1 ml per well). Three days later, 500 μl of medium were removed and the ALL...
cells were seeded on top of the MSC layer. After incubation with cNMPs, NMPs, nucleosides, and/or NBMPR, the cells were separated from the MSC layer by rinsing with a pipet tip. This led to detachment of the ALL cells, while the MSC cells remained adherent. The ALL cells were centrifuged for 5 min at 300×g and then suspended in 100 μl of binding buffer. After that, the cells were incubated with annexin-V-APC for 15 min, followed by an additional incubation step with Pacific Blue anti-human CD3 antibody for 15 min in the dark at ambient temperature. Cells were washed at 300×g for 5 min and then diluted in 300 μl of binding buffer. Apoptosis was determined as described above after addition of PI.

PBMCs were seeded at a density of 1.75 × 10⁵ cells per ml in 1 ml per well on an anti-CD3 antibody-coated 24-well plate with medium containing anti-CD28 antibody. Flow cytometric analysis of apoptosis was performed using the Annexin V/PI method as described above. Similar to the procedure used for the ALL cells, the PBMCs were also stained with Pacific blue-labeled anti-human CD3, and only the cells with the highest fluorescence were gated for flow cytometric analysis of apoptosis.

**HuT-78 cell proliferation assay**

An appropriate number of cells was centrifuged (300×g, 4 min) and resuspended in a minimum of 1 ml of PBS with 0.1% of bovine serum albumin (BSA), yielding a density of 6.7 × 10⁶ cells/ml. After that, an appropriate volume of a 250 μM stock solution of carbocyanine succinimidyl ester (CFSE) was added to yield a final CFSE concentration of 250 nM. The samples were incubated for 10 min at 37 °C, followed by centrifugation (300×g, 4 min) and resuspension in medium to yield a density of 1 × 10⁵ cells/ml. This suspension was seeded in an αCD3-antibody-coated 24-well plate (1 ml/well) and incubated for 72 h (37 °C, 5% CO₂) with cNMPs, NMPs, or nucleosides in the presence or absence of inhibitors (AMP-CP, DPSPX, NBMPR). To counteract potential degradation in case of incubation with AMP-CP or DPSPX, 100 μM of these inhibitors were not only added at the beginning of the incubation time but also after 24 h and 48 h. Thus, after 72 h, the samples contained up to 300 μM of AMP-CP or DPSPX. On the day of the measurement, the content of each well (1 ml) was centrifuged (300×g, 4 min) and resuspended in 1 ml of FACS buffer. Proliferation was analyzed by flow cytometric quantitation of residual cell-bound CFSE fluorescence using a MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany).

**Statistics and data analysis**

Statistical analysis of the data and generation of diagrams was performed with GraphPad Prism 6.07 (GraphPad Software Inc., San Diego, CA, USA). The statistical tests are detailed in the figure legends. All data are provided as means ± SD.

**Results**

**Effect of cyclic nucleotides on anti-CD3 antibody-induced IL-2 production of HuT-78 lymphoma cells**

The effect of 100 μM of the purine cNMPs 3′,5′-cAMP, 3′,5′-cGMP, and 3′,5′-cIMP as well as of the pyrimidine cNMPs 3′,5′-cUMP and 3′,5′-cCMP on αCD3-antibody-induced IL-2 production of HuT-78 lymphoma cells was analyzed by ELISA. Additionally, the corresponding membrane-permeable acetoxyethyl ester analogues (cNMP-AMs) and unmodified cAMP (Fig. 1a) were significantly enhanced by cAMP-AM (Fig. 1b). By contrast, IL-2 production was significantly reduced by unmodified cGMP (Fig. 1a) but was not modulated by the structurally closely related cIMP (Fig. 1a) and also not by cGMP-AM (Fig. 1b). The unmodified pyrimidine cyclic nucleotides cCMP and cUMP (Fig. 1a) as well as their AM derivatives (Fig. 1b) were ineffective.

**Antiproliferative and apoptotic effects of 3′,5′-cGMP and 2′,3′-cGMP and their potential metabolic products on HuT-78 lymphoma cells**

To investigate whether the inhibitory effect of extracellular 3′,5′-cGMP on IL-2 release is associated with a reduction of HuT-78 cell proliferation and/or viability, the influence of 3′,5′-cGMP on proliferation and apoptosis of HuT-78 cells was analyzed in a concentration range from 10 to 200 μM. The experiments were performed in the presence of αCD3 antibody and with an incubation time of 72 h. At baseline, only about 5% of the HuT-78 cells were apoptotic (5.1 ± 1.8%; n = 8 independent experiments; mean ± SD). However, this proportion was significantly raised to 17.7 ± 2.2% and to 21.8 ± 0.6% in the presence of 100 μM and 200 μM of 3′,5′-cGMP, respectively (Fig. 2a). Representative flow cytometric raw data (scattergrams) are depicted in the supplemental information to illustrate the baseline signal (Suppl. Fig. 1a) and the apoptosis in the presence of 100 μM of 3′,5′-cGMP (Suppl. Fig. 1b). Interestingly, a similar pro-apoptotic effect (significant at
100 μM and 200 μM) was also found with the structurally closely related positional isomer 2′,3′-cGMP (Fig. 2b).

We hypothesized that the effects of 3′,5′-cGMP and 2′,3′-cGMP on HuT-78 cell apoptosis could be due to common metabolic products formed by enzymes on the cell surface. To test this hypothesis, the effects of 5′-GMP (potential 3′,5′-cGMP hydrolysis product) and guanosine (possibly formed by degradation of 3′,5′-cGMP and/or 2′,3′-cGMP) were

Fig. 1 Effect of unmodified cNMPs and of membrane-permeant cNMP-AM esters on αCD3-antibody-induced IL-2 production of HuT-78 lymphoma cells. Cells were incubated for 24 h in αCD3-antibody-coated plates with 100 μM of cNMPs (a) or 100 μM of cNMP-AMs (b). The “DMSO” and the “PO₄(AM)₃” bars represent controls for the DMSO content of the cNMP-AM samples and for the intracellular hydrolysis products of the AM esters. Data shown are means ± SD from n = 3 independent experiments. Statistics: one-way ANOVA and Dunnet’s multiple comparison test with medium (a) or PO₄(AM)₃ (b) as control columns. Asterisks indicate significance level: * = p < 0.05; ** = p < 0.01

Fig. 2 Dose-dependent effect of 3′,5′-cGMP, 2′,3′-cGMP, 5′-GMP and guanosine on apoptosis and proliferation of HuT-78 lymphoma cells in the presence of αCD3-antibody. Apoptosis (a-d) and proliferation (e-h) were determined by flow cytometry after 72 h of incubation with increasing concentrations of 3′,5′-cGMP (a, e), 2′,3′-cGMP (b, f), 5′-GMP (c, g), and guanosine (d, h). Apoptosis was determined by combined staining with APC-labeled annexin V and propidium iodide; the anti-proliferative effect of cNMPs was determined via measuring the residual CFSE fluorescence at the end of the incubation time. Please note that increased CFSE fluorescence means reduced proliferation (inverted relationship). Data are means ± SD from n = 3 (2′,3′-cGMP, 3′,5′-cGMP, and 5′-GMP) or n = 4 (guanosine) independent experiments. In all graphs, the data point depicted at log = −5.0 represents the medium control and not a “real” 10 μM concentration. Statistics: one-way ANOVA and Dunnet’s multiple comparison test with medium control (data point at log = −5.0) as control value. Asterisks indicate significance level: * = p < 0.05; ** = p < 0.01; *** = p < 0.001 and **** = p < 0.0001
investigated in apoptosis assays. In fact, both 5′-GMP (Fig. 2c) and guanosine (Fig. 2d) increased apoptosis of HuT-78 cells. This effect reached significance at concentrations of 100 μM and 200 μM. In addition, in later experiments, we also demonstrated that 100 μM of 2′,3′-cGMP (potential metabolite of 2′,3′-cGMP) and 5′-GMP (potential metabolite of 2′,3′- and 3′,5′-cGMP) exerted a highly significant apoptotic effect after 72 h of incubation (Fig. 3b).

Since pro-apoptotic action might be associated with an inhibition of proliferation, we also investigated the effect of the aforementioned guanosine-derived substances on HuT-78 cell proliferation in the CFSE assay. Residual CFSE fluorescence after 72 h of incubation was considerably increased by 100 μM as well as 200 μM of 3′,5′-cGMP, 2′,3′-cGMP, 5′-GMP, and guanosine (Fig. 2e–h), indicating a strong anti-proliferative effect. Due to the large inter-experimental variability, however, the anti-proliferative effect only reached significance in case of 3′,5′-cGMP (at 200 μM), of 5′-GMP (at 200 μM) and of guanosine (at 100 μM and 200 μM) (Fig. 2e–h). In general, the apoptotic and anti-proliferative effects of the investigated guanosine-related compounds started to appear at a concentration of 50 μM, followed by a steep increase of activity between

![Image](https://example.com/image1)

**Fig. 3** Effect of the nucleoside transporter inhibitor NBMPR (1 μM and/or 10 μM) on apoptosis (a, b) and proliferation (c, d) of αCD3-antibody-stimulated HuT-78 cells treated with various guanosine-derived compounds (100 μM). Two independent sets of experiments were conducted. Initially, the experiments included only 10 μM of NBMPR (a, c). Later, 1 μM of NBMPR was additionally included (b, d). For statistical reasons, the two sets were separately depicted and analyzed (two-way ANOVA not possible with partially missing groups). HuT 78 cells were incubated with the test compounds for 72 h in the presence of anti-CD3 antibody (OKT3). The samples contained no inhibitor (open bars) or 1 μM of NBMPR (gray bars) or 10 μM of NBMPR (black bars). Apoptosis (a, b) and proliferation (c, d) were determined by flow cytometry after 72 h of incubation (apoptosis: staining with APC-labeled annexin V and propidium iodide; proliferation: residual CFSE fluorescence at the end of the incubation time, higher residual CFSE fluorescence means less proliferation). Statistical results: Fig. 3a, c: Two-way ANOVA and Dunnet’s multiple comparison test for comparison of guanosine nucleotide effects vs. control column; two-way ANOVA with Sidak’s multiple comparisons test for comparison of inhibitor-containing samples vs. corresponding inhibitor-free controls. Figure 3b, d: two-way ANOVA and Dunnet’s multiple comparison test for comparison of guanosine nucleotide effects vs. control column and for comparison of inhibitor-containing samples vs. corresponding inhibitor-free controls. One, two, three, and four symbols designate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. Significant differences between NBMPR-containing samples and the corresponding inhibitor-free samples are indicated by “*”. Significant differences in comparison to the corresponding control columns are indicated by “+”. Number of independent experiments: a, c: $n = 3$ for both apoptosis and proliferation; b, d: $n = 6$ (apoptosis) and $n = 5$ (proliferation).
50 and 100 μM (Fig. 2a–h). A similarly pronounced anti-proliferative effect was observed in later experiments performed under the same conditions with 100 μM of 2′-GMP (potential metabolite of 2,3′-cGMP) and of 3′-GMP (potential metabolite of 2′,3′- and 3′,5′-cGMP) (Fig. 3d). Interestingly, as shown in Suppl. Fig. 2, 100 μM of the membrane-permeable cGMP-AM also exerted a strong apoptotic effect (Suppl. Fig. 2a) and anti-proliferative (Suppl. Fig. 2b) effect on HuT-78 cells, which is in contrast to its missing or only weak effect on αCD3-induced IL-2 release in Fig. 1 or in Suppl. Fig. 3.

**Inhibition of anti-CD3 antibody-induced IL-2 production by products of 2′,3′-cGMP and/or 3′,5′-cGMP metabolism**

The inhibitory effect of extracellular 3′,5′-cGMP on IL-2 release of HuT-78 cells is associated with reduced HuT-78 cell proliferation and viability. In addition, later experiments have shown that the positional isomer 2′,3′-cGMP as well as the 2′,3′-cGMP/3′,5′-cGMP metabolites 2′-GMP, 3′-GMP, 5′-GMP, and guanosine also exert apoptotic and anti-proliferative effects. This prompted us to investigate whether these metabolites also inhibit IL-2 release. Indeed, ELISA experiments demonstrated that IL-2 release of HuT-78 cells (in the presence of αCD3)-free samples is effectively inhibited by 2′-GMP, 3′-GMP, 5′-GMP, and guanosine (Suppl. Fig. 3).

However, the effects did not reach significance, and the absolute amount of IL-2 released was much lower than in previous experiments (compare Fig. 1 and Suppl. Fig. 3). The “w/o α-CD3” control column in Suppl. Fig. 3 indicates that the HuT-78 cells showed already α-CD3-independent baseline IL-2 production in these experiments, and IL-2 release was not further enhanced by α-CD3 antibody. By contrast, in our previous experiments (Fig. 1), the IL-2 production by HuT-78 cells was so effectively increased by α-CD3 that the control column for α-CD3 was omitted because it was almost zero and did not provide meaningful information. We have no explanation for the large and unpredictable discrepancies in IL-2 release from HuT-78 cells. By contrast, the apoptosis and proliferation measurements showed considerably better inter-experimental reproducibility. For this reason, we focused on apoptosis and proliferation assays for the rest of the project.

**Role of equilibrative nucleoside transporters in mediating cytotoxicity of guanosine-derived compounds**

We hypothesized that both 3′,5′-cGMP and 2′,3′-cGMP are first converted to mononucleotides by an ecto-phosphodiesterase on the cell surface, followed by the formation of guanosine by ectonucleotidases. This would mean that guanosine is the common anti-proliferative and pro-apoptotic metabolic end-product of 3′,5′-cGMP, 2′,3′-cGMP, 5′-GMP, 3′-GMP, and 2′-GMP. Guanosine may then enter HuT-78 cells via a nucleoside transporter and unfold its cytotoxic effects from inside the cell.

To test this hypothesis, HuT-78 cells were incubated with guanosine and guanosine nucleotides in the presence and absence of S-(4-Nitrobenzyl)-6-thioinosine (NBMPR), which preferentially inhibits the human equilibrative nucleoside transporter hENT1. According to the literature, the IC50 value for nucleoside transport inhibition by NBMPR is 0.4 nM and 2.8 μM for hENT1 and hENT2, respectively (Ward et al. 2000). Thus, we used a concentration of 10 μM of NBMPR, which should be sufficient to completely inhibit guanosine uptake by hENT1 and which should only partially block hENT2. Again, 100 μM of guanosine, 5′-GMP, 2′,3′-cGMP, or 3′,5′-cGMP significantly promoted apoptosis (Fig. 3a) and inhibited proliferation (Fig. 3c) (two-way ANOVA with Sidak’s multiple comparisons test, comparisons with corresponding control column). The hENT1 inhibitor NBMPR was clearly protective and inhibited the pro-apoptotic and anti-proliferative actions of the tested guanosine-derived compounds (Fig. 3a, c). The protective NBMPR effect reached significance for 2′,3′-cGMP, 3′,5′-cGMP, and guanosine in the apoptosis experiments (Fig. 3a) and for 2′,3′-cGMP and guanosine in the proliferation assay (Fig. 3c) (two-way ANOVA with Sidak’s multiple comparisons test for comparison of inhibitor-containing samples with corresponding inhibitor-free controls). Later in the project, we performed a second set of experiments that included not only 10 μM of NBMPR but also the lower concentration of 1 μM. Even at a concentration of only 1 μM, NBMPR protected HuT-78 cells from the cytotoxic effects of 100 μM of the 2′,3′-cGMP and/or 3′,5′-cGMP metabolites 2′-GMP, 3′-GMP, 5′-GMP, and guanosine. This is illustrated for apoptosis in Fig. 3b and for proliferation in Fig. 3d. Interestingly, Fig. 3b, d show that NBMPR has already a maximum protective effect at a concentration of 1 μM. By contrast, 10 μM of NBMPR seem to be slightly cytotoxic, as indicated by a weak apoptotic and anti-proliferative effect (Fig. 3b, d).

Moreover, NBMPR counteracted the inhibitory effect of 2′-GMP, 3′-GMP, 5′-GMP, and guanosine on IL-2 release, although the effects did not reach significance (ELISA data in Suppl. Fig. 3). Suppl. Fig. 3 again indicates that 1 μM of NBMPR is already sufficient to cause the maximum protective effect in the IL-2 assay.

**Determination of the IC50 value of NBMPR**

The results reported so far suggest that 2′,3′-cGMP and 3′,5′-cGMP are metabolized to the end-product guanosine, which is taken up into the cell by an NBMPR-sensitive nucleoside transporter and then exerts apoptotic and anti-proliferative effects. The two most important nucleoside transporters for guanosine, ENT1 and ENT2, show largely different IC50 values for NBMPR (see Table 1: 0.4 nM for ENT1 and 2.8 μM for...
ENT2). A third, yet undefined transporter is responsible for the csg guanosine transport process and is inhibited by NBMPR with a $K_i$ value of 0.7 nM.

To learn more about the identity of the transporter responsible for the observed guanosine effects in HuT-78 cells, we recorded concentration-effect curves for apoptosis (Fig. 4a) and proliferation (Fig. 4b) with increasing concentrations of NBMPR in the presence of 100 μM of guanosine (red curves in Fig. 4). Since NBMPR exerts an apoptotic and anti-proliferative effect by itself at higher concentrations (cf. data shown in the preceding section), we additionally determined a concentration-effect curve in the absence of guanosine (black curves in Fig. 4). This background effect of NBMPR alone was subtracted from the guanosine + NBMPR data, yielding the net effect of guanosine in the presence of increasing NBMPR concentrations (blue curves in Fig. 4). The IC$_{50}$ value of NBMPR was ~ 25 nM in the apoptosis assays and ~ 28 nM in the proliferation experiments.

### Role of ecto-phosphodiesterase and ecto-5′-nucleotidase in mediating cytotoxicity of guanosine nucleotides

After having shown that NBMPR-sensitive nucleoside transport is the common mechanism for the cytotoxic effects of guanosine and guanosine-derived nucleotides, we addressed the steps that may lead to guanosine formation from the corresponding cyclic nucleotides and mononucleotides. First, we investigated the effect of DPSPX (1,3-dipropyl-8-sulfophenylxanthine), which is not only a well-known adenosine receptor antagonist but also an inhibitor of ecto-phosphodiesterases. Cells were incubated with 100 μM of DPSPX during the first 24 h. After 24 h and 48 h, 100 μM of fresh DPSPX were added to compensate for potential hydrolytic inactivation. Thus, in the absence of DPSPX hydrolysis, the DPSPX concentration in the samples would reach 300 μM after 72 h. However,

### Table 1: Important transport processes for nucleosides and nucleoside analogues

| Process                  | Protein | Expression                                      | Examples for physiological substrates ($K_m$)                          | Examples for nonphysiological substrates ($K_m$)                              | NBMPR sensitive? ($IC_{50}$) |
|--------------------------|---------|------------------------------------------------|------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------|
| Equilibrative, bidirectional facilitators (ENT transporter family: SLC29) |         |                                                |                                                                        |                                                                            |                             |
| $es$                     | hENT1   | ubiquitous                                     | adenosine (50 μM), guanosine (140 μM), inosine (200 μM), uridine (480 μM), thymidine (240 μM), cytidine (680 μM) | gemcitabine (160 μM), cytarabine, fludarabine, cladribine                   | yes (0.4 nM)                |
| $ei$                     | hENT2   | ubiquitous, high abundance in skeletal muscle  | adenosine (140 μM), guanosine (270 μM), inosine (50 μM), uridine (270 μM), thymidine (620 μM), cytidine (5210 μM) | gemcitabine (740 μM), cladribine, cytarabine, fludarabine                   | no (2.8 μM)                 |
| –                        | hENT3   | ubiquitous, e.g. placenta, mainly intracellular, optimum activity at pH 5.5 | adenosine (1900 μM), uridine (2000 μM)                                  | e.g. gemcitabine                                                           | no                          |
| –                        | hENT4 (PMAT) | ubiquitous                                    | adenosine (780 μM), organic cations including serotonin (1900 μM)       | 1-methyl-4-phenylpyridinium (MPP+), neurotoxin                               | no                          |
| Concentrative, inwardly directed sodium/nucleoside cotransporters (CNT transporter family: SLC28) |         |                                                |                                                                        |                                                                            |                             |
| $cit$                    | hCNT1   | e.g. epithelial tissues of small intestine, kidney and liver | adenosine, pyrimidine nucleosides                                     | zidovudine, lamivudine, gemcitabine (17 μM), cytarabine, 5'-DFUR (209 μM) | no                          |
| $cif$                    | hCNT2   | numerous tissues, e.g. kidney, liver, heart, brain, placenta, pancreas | purine nucleosides, uridine                                           | didanosine, ribavirin                                                      | no                          |
| $cib$                    | hCNT3   | e.g. pancreas, trachea, bone marrow, mammary gland | purine and pyrimidine nucleosides                                     | gemcitabine, fludarabine, cladribine                                        | no                          |
| $csg$                    | ???     | NB4 promyelocytic leukemia cells, L1210 murine acute lymphocytic leukemia cells | guanosine                                                              |                                                                             | yes (0.7 nM)                |

The information in this table comes from the following publications: (Flanagan and Meckling-Gill 1997; Ward et al. 2000; Young et al. 2008; Pastor-Anglada et al. 2004; Gray et al. 2004; Govindarajan et al. 2009). Please note that this table is not exhaustive and does not completely cover the literature on substrate selectivity of nucleoside transporters. Abbreviations: PMAT = Plasma membrane monoamine transporter; 5'-DFUR = 5'-Deoxy-5-fluorouridine.
DPSPX did not inhibit the pro-apoptotic (Fig. 5a) and anti-proliferative (Fig. 5b) effects of 2′,3′-cGMP, 3′,5′-cGMP, 5′-GMP, and guanosine. Second, we analyzed whether AMP-CP (adenosine 5′-(α,β-methylene)diphosphate), an inhibitor of the ecto-5′-nucleotidase CD73, was able to modulate the effects of 5′-GMP on HuT-78 cell apoptosis and proliferation. Since guanosine would not require CD73 for its activation, it was selected as a negative control. Even after 72 h of incubation with 100 μM of AMP-CP, no alteration of the pro-apoptotic (Fig. 6a) or anti-proliferative (Fig. 6b) effects of 5′-GMP (black bars in Fig. 6a, b) and guanosine (gray bars in Fig. 6a, b) was observed. To compensate for potential hydrolysis of AMP-CP, other samples were run in parallel, where 100 μM of fresh AMP-CP was added after 24 h and 48 h (similar as described above for DPSPX; bars labeled with “AMP-CP (daily)” in Fig. 6a, b). However, no effect of AMP-CP was observed in these samples either.

As shown in Fig. 1, unlike 3′,5′-cGMP, the structurally closely related cyclic nucleotide 3′,5′-cIMP had no influence on αCD3-antibody-induced IL2-production. Therefore, we investigated if this also pertains to apoptosis and proliferation. In fact, neither 100 μM nor 200 μM of 3′,5′-cIMP altered apoptosis (Fig. 7a) or proliferation (Fig. 7c) of HuT-78 cells. The other purine cyclic nucleotide, 3′,5′-cAMP, was also ineffective at 100 μM in both readouts (Fig. 7a, c), confirming the results from the IL2 ELISA experiments. Only at a concentration of 200 μM, 3′,5′-cAMP exerted a highly significant apoptotic and anti-proliferative effect (apoptosis: \( p < 0.0001 \); proliferation: \( p < 0.01 \); compared with medium control; two-way ANOVA with Dunnet’s multiple comparison test; Fig. 7a, c). By contrast, the positional isomer 2′,3′-cAMP was neither pro-apoptotic nor anti-proliferative (Fig. 7b, d).
Adenosine, a potential metabolic product of both 2′,3′- or 3′,5′-cAMP, was inactive with regard to proliferation (Fig. 7d) but significantly active at 200 μM in the apoptosis assay ($p < 0.01$ compared with medium control; two-way ANOVA after 72 h of incubation. Apoptosis was determined by combined staining with APC-labeled annexin V and propidium iodide; the antiproliferative effect of cNMPs was determined via measuring the residual CFSE fluorescence at the end of the incubation time. Higher residual CFSE fluorescence means less proliferation. Statistics: asterisks indicate significant effect of treatment with guanosine-derived compounds (two-way ANOVA and Dunnet’s multiple comparison test, comparisons with corresponding control column). Data are means ± SD from $n = 4$ independent experiments with Dunnet’s multiple comparison test; Fig. 7b). The cyclic pyrimidine nucleotides 3′,5′-cCMP and 3′,5′-cUMP that had been ineffective at inhibiting αCD3 antibody-induced IL2-release were also completely inactive with respect to apoptosis

![Fig. 6](image-url)  
**Fig. 6** Effect of the 5′-ectonucleotidase (CD73) inhibitor AMP-CP on the pro-apoptotic (a) and anti-proliferative (b) actions of 100 μM of 5′-GMP or guanosine (HuT-78 lymphoma cells in the presence of αCD3 antibody). Cells were incubated with guanosine-derived compounds (black bars: 5′-GMP; gray bars: guanosine) in the absence or presence of 100 μM of the ecto-5′-nucleotidase inhibitor AMP-CP as designated in the x-axis labeling. The inhibitor was either added only once in the beginning at $t = 0$ h, or it was added freshly every day (at $t = 0$ h, 24 h, and 48 h; indicated as “AMP-CP (daily)” in the labeling of the bars). In both cases, the total incubation time lasted 72 h. Apoptosis (a) and proliferation (b) were determined by flow cytometry. Apoptosis was determined by combined staining with APC-labeled annexin V and propidium iodide; the anti-proliferative effect was determined via measuring the residual CFSE fluorescence at the end of the incubation time. Higher residual CFSE fluorescence means lower proliferation. Data are means ± SD from $n = 2$ independent experiments.
and proliferation (Fig. 7c), even at a concentration of 200 μM. Likewise, the positional isomers 2′,3′-cCMP and 2′,3′-cUMP did not influence the readouts for apoptosis (Fig. 7b) and proliferation (Fig. 7d).

Cytotoxicity of guanosine and guanosine-related compounds on PBMCs/T cells and acute lymphoblastic leukemia (ALL) xenografts

Since HuT-78 cells are lymphoma cells and the properties of cancer cell lines frequently differ from those of primary cells, we conducted additional experiments with freshly isolated PBMCs that also contain T lymphocytes. Since the PBMCs were incubated throughout the experiment (i.e., for 72 h) in the presence of anti-CD3 (OKT3) and anti-CD28 antibody, the proportion of T cells was further increased. In fact, these cells behaved very differently. Unlike in HuT-78 cells, none of the guanosine-related compounds induced apoptosis (Fig. 8a; open bars). Consequently, NBMPR (10 μM) did also not modulate any of these readouts in PBMCs (Fig. 8a; filled bars).

Since guanosine-related compounds were only active in HuT-78 cells but not in PBMCs, they may have therapeutic potential for the treatment of T cell lymphomas. Such compounds may selectively kill lymphoma cells without affecting the healthy T cell population. To elucidate whether the observed apoptotic and anti-proliferative effects also pertain to other lymphomas, we investigated the effect of 100 μM of 2′,3′-cGMP, 3′,5′-cGMP, 5′-GMP, and guanosine on cortical T cell acute lymphoblastic leukemia (ALL) xenograft cells. Adenosine (100 μM) was also investigated as a “non-guanosine negative control.” The xenografts had been previously produced by growing patient-derived tumor cells in immunologically deficient NSG mice (Frismantas et al. 2017). In contrast to conventional cancer cell lines, xenografts have the advantage that their characteristics are very close to primary cancer cells since they retain the pattern of mutations present in the
original patient-derived material (Frismantas et al. 2017). Our experiment was conducted under similar conditions as used for the HuT-78 cells on anti-CD3-antibody-treated plates. However, unlike the HuT-78 cells, the xenograft cells were cultured in the presence of feeder cells (mesenchymal stem cells, MSC) as described by Frismantas et al. (2017). Moreover, a second set of wells was used to perform the experiment in the absence of anti-CD3 coating. Figure 8b shows that basal apoptosis under control conditions was already relatively high (40–50%) but independent of the presence of anti-CD3 antibody. Control wells without MSC feeder cells showed almost 100% apoptosis, indicating that MSC cells are absolutely necessary for the viability of the xenograft cells. The guanosine-derived compounds had no significant effect. Only a weak pro-apoptotic trend was observed (increase by about 10%, Fig. 8b). However, 10 μM of NBMPR did not protect the cells, when combined with 100 μM of guanosine (Fig. 8b). Interestingly, the lowest apoptosis rate was observed in the presence of 100 μM of adenosine, suggesting a protective effect.

Discussion

Apoptotic and anti-proliferative effects of guanosine-related compounds

Prompted by the observation that 3′,5′-cGMP inhibits αCD3 antibody-stimulated IL-2 production of HuT-78 T-lymphoma cells, we have performed a detailed investigation of the effects of guanosine-related compounds on HuT-78 cell apoptosis and proliferation. We have mainly focused on apoptosis and proliferation data because they showed lower inter-experimental variability than anti-CD3 antibody-stimulated IL-2 production. The results presented in this publication demonstrate that guanosine as well as the guanosine-related nucleotides 2′,3′-cGMP, 3′,5′-cGMP, 2′-GMP, 3′-GMP, and 5′-GMP increase apoptosis and reduce proliferation of HuT-78 T lymphoma cells. It is unlikely that these effects are caused by activation of adenosine receptors because adenosine was active in apoptosis assays only at a high concentration of 200 μM and completely ineffective in proliferation experiments. Moreover, DPSPX, which is not only an ecto-phosphodiesterase inhibitor but also a non-selective adenosine receptor antagonist, did not eliminate the effects of the tested guanosine-related compounds. We developed several hypotheses to explain our observations (Fig. 9).

First hypothesis: guanosine is formed extracellularly and taken up into HuT-78 cells by an NBMPR-sensitive nucleoside transporter

First, we hypothesized that the cytotoxic effects of 2′,3′/3′,5′-cGMP as well as 2′-GMP, 3′-GMP and 5′-GMP are caused by guanosine, which is formed by enzymatic breakdown of the nucleotides and enters the cells through NBMPR-sensitive nucleoside transporters. The guanosine-cNMPs could be hydrolyzed to mononucleotides by ecto-phosphodiesterases (Fig. 9, step 1), followed by the formation of guanosine after further ectonucleotidase-mediated digestion (Fig. 9, step 2). In fact, metabolic pathways that lead to the formation of...
guanosine from cyclic guanine nucleotides have been described for 3′,5′-cGMP (Albrecht et al. 2013) and, very recently, for 2′,3′-cGMP (Jackson et al. 2019). A "2′,3′-cGMP-guanosine pathway" (Jackson et al. 2019) has been suggested in analogy to the previously described "2′,3′-cAMP-adenosine pathway" (Verrier et al. 2011; Jackson 2011).

Unexpectedly, the unselective ecto-phosphodiesterase inhibitor DPSPX did not eliminate the cytotoxic effects of 2′,3′- or 3′,5′-cGMP, and the ecto-5′-nucleotidase inhibitor AMP-CP did not counteract the apoptotic and anti-proliferative action of 5′-GMP. It is unlikely that these inhibitors were inactivated by hydrolysis during the 72 h incubation time because we did not even observe an effect when fresh AMP-CP or DPSPX were re-added after 24 h and 48 h. Thus, if guanosine was really formed from the guanosine-derived nucleotides in our experiments, the corresponding mechanism remains elusive.

The guanosine generated by guanosine nucleotide metabolism may be taken up by the cells through NBMPR-sensitive nucleoside transporters (Fig. 9, step 3) and then cause apoptotic and anti-proliferative effects by intracellular action (Fig. 9, step 4). It is well-known that extracellular nucleosides can be taken up by cells through specific transporters. As shown in Table 1, the various nucleoside transport processes can be divided in sodium-independent equilibrative (ENT) and sodium-dependent concentrative (CNT) processes. The ENT processes are bidirectional and comprise the es and the ei process. The hENT1 molecule is basically responsible for es, while the hENT2 transporter shows ei activity.

In our experiments with HuT-78 cells, 10 μM of NBMPR, an inhibitor of the human equilibrative nucleoside transporters hENT1 (IC₅₀ = 0.4 nM) and hENT2 (IC₅₀ = 2.8 μM), completely eliminated the cytotoxic effects of guanosine and guanosine-derived nucleotides. Additional experiments indicated that even 1 μM of NBMPR is already sufficient for the full protective effect. Concentration-effect curves with 100 μM of guanosine alone or in combination with increasing concentrations of NBMPR resulted in NBMPR IC₅₀ values of 25 nM (apoptosis) and 28 nM (proliferation). The Cheng-Prusoff equation (Cheng and Prusoff 1973) \( K_{I} = IC_{50}/(1 + [S]/K_{M}) \) was employed with [S] being the concentration of the substrate guanosine (100 μM) and K₅₀ representing the guanosine K₅₀ value. Using the K₅₀ value of guanosine for hENT1 for the calculation...
(140 μM, Table 1) yielded NBMPR Kᵢ values of ~14.6 nM (apoptosis) and of 16.3 nM (proliferation). This is still ~40-fold higher than the literature NBMPR Kᵢ (high-affinity [³⁰H]NBMPR binding) at hENT1 (0.38 nM; Ward et al. 2000), which may be due to the fact that we did not determine the direct effect of NBMPR on guanosine transporter activity but used an indirect downstream parameter (apoptosis or proliferation).

Alternatively, according to our second hypothesis, intracellularly and exported by an NBMPR-sensitive transporter, possibly hENT1 (Fig. 9, step 7). After that, guanosine may cause apoptosis by extracellular action, e.g. via putative guanosine receptors or other yet to be defined target sites (Fig. 9, step 8). Not much is known about guanosine receptors. Several years ago, a G protein-coupled guanosine receptor has been postulated in the rat brain on the basis of data from [³⁰H]guanosine radioligand binding assays (Traversa et al. 2003, 2002), cAMP accumulation assays (Traversa et al. 2003), or europium-based Gox-activation assays (Volpini et al. 2011). However, to the best of our knowledge, the molecular identity of this binding site is still elusive.

Since OATs are largely NBMPR insensitive, the second hypothesis would explain the inhibitory effect of NBMPR by a reduction of guanosine export from the cells. However, since this hypothesis assumes extracellular action of guanosine, it cannot explain why extracellularly applied guanosine acts in an NBMPR-sensitive way. Perhaps reality comprises a mixture of mechanisms from the first and the second hypothesis.

**Different effects of cGMP and cGMP-AM on IL-2 release and apoptosis/proliferation**

The ELISA data in Fig. 1 show that cGMP-AM does not affect IL-2 release, while 3′,5′-cGMP has a pronounced inhibitory effect. By contrast, a weak and non-significant inhibitory effect of cGMP-AM on IL-2 release was visible in later experiments (Suppl. Fig. 3). It is noted, however, that the data in Suppl. Fig. 3 show large variability and the IL-2 release is only very low and not influenced by αCD3. Thus, the results regarding the cGMP-AM effect on IL-2 production are rather inconclusive in Suppl. Fig. 3. By contrast, cGMP-AM had a clear pro-apoptotic and anti-proliferative effect (Suppl. Fig. 2), which was comparable with the cytotoxic effects of the other tested guanosine-related compounds.

We assume that the difference in cGMP-AM effect between IL2 ELISAs and apoptosis/proliferation experiments is due to the longer incubation time of 72 h in the apoptosis/proliferation assays as compared to only 24 h in the ELISA experiments. Previous pilot experiments (data not shown) indicated that it took 72 h for 5′-GMP and guanosine to produce pronounced effects on apoptosis and proliferation. By contrast, IL-2 production by HuT-78 cells may follow a different time course. Moreover, intracellular delivery of cGMP from cGMP-AM may be more important during the first 24 h of incubation. By contrast, during longer incubation times, hydrolysis of cGMP-AM in the medium may produce large amounts of extracellular cGMP, which makes it difficult to discriminate between intracellular and extracellular actions of cGMP derived from cGMP-AM. The difference between the effects of cGMP and cGMP-AM and the underlying mechanisms should be investigated in more detail in future studies.

**Pathophysiological roles of extracellular cGMP**

In our experiments, we have added extracellular cGMP to HuT-78 cell cultures. At first glance, this seems to be an odd...
way to investigate cGMP effects, as cGMP is widely recognized as an intracellular second messenger. However, (patho)physiological roles of extracellular cGMP have been demonstrated in the past. Suppl. Table 1 shows several examples of extracellular cGMP effects reported in the literature. Extracellular cGMP modulates natriuresis in the kidneys. It seems to interfere with the effect of various platelet-activating agents. Moreover, extracellular cGMP appears to act in the gastrointestinal tract, e.g. by modulating fluid absorption and secretion as well as visceral sensitivity. Numerous effects of extracellular cGMP have been reported in the CNS, mainly in animal models of hepatic hyperammonemic encephalopathy. The references in Suppl. Table 2 suggest that the effect of extracellular cGMP seems at least partly mediated by conversion to guanosine. However, Suppl. Tables 1 and 2 show mostly neuroprotective effects of extracellular cGMP and guanosine, while our results rather support apoptotic and anti-proliferative action of extracellular cGMP in HuT-78 cells. This difference suggests that extracellular cGMP exerts very distinct cell type-dependent effects.

Nucleoside transporters and nucleoside-derived cytostatic drugs

Nucleoside transporters are an important prerequisite for the activity of nucleoside-derived drugs, e.g., several anti-cancer agents. The NBMPR-sensitive transporter ENT1 mediates uptake of the cytostatic drugs gemcitabine, fludarabine, cladribine, and cytarabine (Table 1) (Pastor-Anglada et al. 2004). CEM lymphoblastic leukemia cells express ENT1 and are sensitive to gemcitabine (Mackey et al. 1998). However, in the presence of NBMPR, the IC50 of gemcitabine increased by more than a 100-fold (Mackey et al. 1998). Moreover, it has been recently reported that the imidazole nucleoside immunosuppressant mizoribine is taken up by L5178Y-R mouse lymphoma cells and metabolized to the corresponding monophosphate. The L5178Y-R cells express mRNA for both ENT1 and ENT2. Mizoribine uptake was inhibited by the unselective ENT1/ENT2 substrate adenosine but not by 0.1 μM of the ENT1 inhibitor NBMPR (Oda et al. 2018).

Our results suggest that guanosine and/or guanosine-related compounds could be an important addition to the cytostatic therapy of specific kinds of leukemia. The efficacy, however, may be highly dependent on the presence of the corresponding nucleoside transporters, e.g. ENT1 or csg. This has been demonstrated for several established cytostatic drugs. For example, CEM-ARA-C/8C cells are virtually resistant to gemcitabine because they are deficient of any kind of nucleoside transport (Mackey et al. 1998). Later, it has been reported that CEM-ARA-C/8C cells still express ENT1, but a glycine-to-arginine mutation in the ENT1 protein (G24R) results in a loss of nucleoside uptake activity (Zimmerman et al. 2009). Moreover, it has been proposed that immunohistochemical determination of hENT1 expression may be useful to predict gemcitabine or capecitabine resistance of breast cancer cells (Mackey et al. 2002).

Lack of nucleoside transporter expression in some cancers may cause resistance to nucleoside-derived cytostatic drugs. This may be the reason, why guanosine and guanosine-related compounds were practically ineffective in our experiments with ALL xenograft cells. In fact, it has been reported that ENT transporter expression in fresh leukemic lymphoblasts isolated from four different ALL patients showed considerable inter-individual variability that was reflected by very heterogeneous cytostatic effects of cytarabine (Gati et al. 1998). It should be noted, however, that no information about the exact type of ALL was provided by Gati et al. (1998). We hypothesize that the ALL clone used in our experiments may have shown a rather low expression of guanosine-transporting proteins. Thus, as previously recommended for breast cancer (Mackey et al. 2002), also in case of different kinds of leukemia, a pre-screening for nucleoside transporter expression may help to select patients responsive to therapy with nucleoside-derived cytostatics. In this regard, it should be noted that there may be other, NBMPR-insensitive, transport processes that are able to mediate guanosine uptake. For example, guanosine increased apoptosis of Jurkat (human T cell leukemia) cells in an NBMPR-insensitive way (Batiku et al. 2001).

Nucleoside transporters in PBMCs

We can only speculate why guanine nucleotides and guanosine did not affect viability and proliferation of PBMCs in our experiments. Since we have cultured the PBMCs during our experiments for 72 h in the presence of αCD3- and αCD28 antibody, we assume that proliferation of T cells was selectively stimulated and T cells were enriched in our culture. Very recently, it has been demonstrated that peripheral T cells show very high abundance of mRNA for ENT3 but only very low amounts of ENT1 or ENT2 mRNA (Wei et al. 2018). ENT3, however, is mainly expressed intracellularly in lysosomal and mitochondrial membranes (Govindarajan et al. 2009) and therefore unable to mediate uptake of extracellular nucleosides. By contrast, another publication reports hENT1 mRNA in PBMCs, but experiments with tritiated [3H]gemcitabine revealed that the concentrative nucleoside transporter hCNT1 contributed much more to [3H]gemcitabine uptake than hENT1 (Choi 2012). To the best of our knowledge, hCNT1 mainly accepts pyrimidine nucleosides as substrates and no guanosine transport by hCNT1 has been reported till now (Pastor-Anglada et al. 2004). Alternatively, PBMCs could take up guanosine by other transporters (e.g. hCNT2 or hCNT3) but may be resistant to the apoptotic effects of guanosine.
Conclusion and outlook

We have identified apoptotic and anti-proliferative effects of guanosine and guanosine-related compounds in HuT78 human Sézary lymphoma cells. These effects seem to strongly depend on the activity of an NBMPR-sensitive nucleoside transporter, the identity of which is still elusive. Our concentration effect curves with NBMPR suggest that ENT2 is most likely not involved. We propose that guanosine and related compounds could serve as potential adjuvants for the treatment of various kinds of guanosine transporter-positive cancers, in addition to established cytostatic nucleoside analogues.

Future studies will have to clarify if HuT-78 cells take up guanosine via the \(\sigma\) (ENT1) and/or the \(\sigma\)g process. Moreover, other cancer cell lines should be tested for their guanosine sensitivity, and the data should be correlated with expression levels of ENT1 (e.g. as determined by \[^3\text{H}\]NBMPR binding) or with the guanosine uptake capacity (e.g. uptake of \[^3\text{H}\]guanosine). Moreover, it should be investigated whether extracellular effects of guanosine play a role in some cell lines, e.g. cytotoxicity mediated by adenosine receptors (Oliveira et al. 2017). Finally, the effect of guanosine should be investigated in mouse leukemia or lymphoma models since the guanosine-accepting \(\sigma\) nucleoside transport process (probably caused by ENT1) was detected in murine lymphoma (S49) and leukemia (L1210) cancer cell lines (Mackey et al. 1998).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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