Involvement of MAP Kinases in the Cytotoxicity of Acyclic Nucleoside Phosphonates

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Abstract. *Background: 9-[2-(phosphonomethoxy)ethyl] guanine (PMEG) is a nucleotide analogue with anticancer activity. Here we investigate the role of ERK, p38, JNK and AKT kinases in PMEG-induced apoptosis. Materials and Methods: CCRF-CEM and HL-60 leukemia cells were used to assess MAPK mRNA and protein expression in PMEG-treated cells. MAPK activation was measured using phospho-specific antibodies. Apoptosis was evaluated by caspase-3 and PARP cleavage. Results: Up-regulation of p38β, γ and δ mRNA were observed following PMEG treatment of CCRF-CEM cells, however, the total protein expression remained unchanged. Neither PMEG nor its analogue 9-[2-(phosphonomethoxy) ethyl]-2,6-diaminopurine (PMEDAP) induced p38 kinase phosphorylation in CCRF-CEM cells, whereas increased p38 phosphorylation was observed in HL-60 cells. The ERK pathway was also activated by these compounds. Pretreatment of the cells with the p38 inhibitor SB203580 diminished drug-induced apoptosis whereas inhibition of ERK, JNK or AKT pathways did not dose. Conclusion: PMEG- and PMEDAP-induced cytotoxicity is partly mediated by the p38 pathway in human leukemia cells.*

The mitogen-activated protein kinases (MAPK) are involved in three Ser/Thr kinase cascades. Extracellular-signal-related kinases (ERKs) respond to growth factors or other mitogenic signals by promoting cell proliferation. The other two pathways – p38 MAPK and the c-Jun N-terminal kinase (JNK) promote stress responses such as inflammation or programmed cell death (1, 2). Protein kinase B (AKT) represents another Ser/Thr kinase involved in the regulation of cell survival, differentiation and division and its pathway (PI3K/AKT/mTOR) is frequently activated simultaneously with the one of ERK kinase (RAF/MEK/ERK) (3). Constitutive activation of MAPK survival signaling leads to increased cell proliferation and tumor progression (4) while MAPK was also suggested to determine sensitivity of the cells to chemotherapeutic agents (5). These findings boosted research of inhibitors targeting ERK or AKT pathways as a novel strategy on how to eliminate cancerous cells or at least on how to sensitize tumors to chemotherapy (6, 7).

9-(2-Phosphonomethoxyethyl) guanine (PMEG) and 9-(2-phosphonomethoxyethyl) diaminopurine (PMEDAP) are acyclic nucleoside phosphonate (ANP) analogs possessing significant anticancer activities both *in vitro* and *in vivo* (8, 9). PMEG prodrug GS-9219 is being developed as a chemotherapeutic for lymphomas and certain types of leukemia (10, 11). To date, the question whether MAPKs may be involved in PMEG/PMEDAP-induced cytotoxicity towards leukemia cells has not been addressed. The aim of this work was to evaluate the effects of cytotoxic acyclic nucleoside phosphonates (PMEG, PMEDAP) on the expression and activation of ERK, p38, JNK and AKT kinase in leukemia cells and to elucidate whether these kinases participate in the apoptotic effects of PMEG and PMEDAP. We also investigated the potential inhibitory activities of various ANPs towards ERK and AKT kinase.

Materials and Methods

Materials. PMEG and PMEDAP were prepared according to the previously published procedures (12). Streptomycin, penicillin G and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA), fetal calf serum was obtained from PAA Laboratories GmbH (Pasching, Austria). All antibodies were furchased from Cell Signaling Technology (Danvers, MA, USA). SuperSignal® West Femto Maximum Sensitivity Chemiluminescent Substrate and Restore™ Western Blotting Stripping Buffer were from Pierce (Rockford, IL, USA).

Cell culture and proliferation assay. CCRF-CEM cells (ATCC CCL-119) and HL-60 cells (ATCC CCL-240) were obtained from LGC standards (Brno, Czech Republic). They were cultured under

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a humidified atmosphere containing 5% CO_2 at 37˚C in RPMI-1640 medium supplemented with 10% fetal calf serum, 200 μg/ml of streptomycin, 200 U/ml of penicillin G and 4 mM glutamine. Cells were seeded into the culture flasks at a concentration of 100,000 cells/ml and left to attach for 24 h. Cells were then pretreated with 10 μM of ERK, JNK, p38 or AKT inhibitors for 30 min after which the indicated concentration of PMEG or PMEDAP was added and cells were cultured for additional 24-72 h. Proliferation was assessed using Countess® Automated Cell Counter (Invitrogen, San Diego, USA) following Trypan blue (0.4% w/v) staining 1:1.

Quantitative reverse transcription PCR. Total RNA from 10^6 treated or untreated cells was extracted using RNeasy Mini isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 0.5 μg RNA using RT^2 First Strand kit (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. Human MAPK RT^2-Profiler PCR array (SABiosciences) was then used for gene quantification employing DNA Engine Opticon® 2 thermocycler (Biorad, Hercules, CA, USA). The expression of p38 kinase isoforms was confirmed with RNA from CCRF-CEM and HL-60 cells, treated with different concentrations of PMEG and PMEDAP. PCR was performed using SYBR Green master mixes and validated PCR primers (SABiosciences).

Immunoblotting. Cells (10^7) were lysed in 150 μl of RIPA buffer containing protease and phosphatase inhibitors (Pierce). The 30 μg of total protein were separated by 12% polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.05% of Tween 20 (Pierce) and probed with anti-ERK, anti-p38, anti-JNK, anti-AKT, anti-p-ERK, anti-p-38, anti-p-JNK, anti-p-AKT or anti-β-actin and an appropriate HRP-conjugated IgG. Chemiluminescent signal was captured by a CCD camera. The membrane was first probed with the phospho-specific antibody, then stripped and reprobed with the phosphorylation-insensitive antibody. β-Actin was used to ensure uniform protein loading.

In vitro kinase assays. Recombinant active ERK2 (100 ng) (Merck, Darmstadt, Germany) was incubated with 0.3 μCi [γ-^{32}P]ATP (167 TBq/mmol, MP Biomedicals, Inc., Irvine, CA, USA) and 0.4 mg/ml myelin basic protein (Merck) in 30 μl of kinase buffer: 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EGTA, 10 mM dithiothreitol, 10 mM $MgCl_2$, 100 μM ATP. Reactions were incubated at 30˚C for 30 min and terminated by spotting a 2-μl aliquot onto a phosphocellulose disc (Whatman P-81). The air-dried discs were then washed 3 × 5 min in 0.5% phosphoric acid and 1 × 5 min in acetone. The radioactivity was counted on a TriCarb 2900TR beta-counter (Perkin Elmer, Waltham, MA, USA). The reaction mixture for AKT kinase activity determination consisted of 100 ng of recombinant AKT kinase (Merck), 50 μM AKTide-2T (synthetic peptide substrate, Merck) and 0.3 μCi [γ-^{32}P]ATP in 30 μl of the same buffer as the one used for the ERK kinase assay.

Data analysis. Unless otherwise indicated data are means±SD of three independent experiments. Statistical evaluation was performed with use of GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Table I. *MAPK gene expression profiling in CCRF-CEM cells following treatment with 1 μM 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG) for 24 and 72 hours (n=2).*

	Fold change	
	24 h	72 h
MAPK1 (ERK/ERK2)	1.0	1.3
MAPK3 (ERK1)	1.1	1.1
MAPK8 (JNK1)	1.1	1.2
MAPK9 (JNK2)	1.0	1.1
MAPK10 (JNK3)	1.0	1.5
MAPK11 (p38β)	1.4	2.1
MAPK12 (p38γ)	1.7	1.8
MAPK13 (p38δ)	1.1	2.8

ERK: Extracellular-signal-related kinase; JNK: c-Jun N-terminal kinase.

Results

Distinct modulation of p38 MAPK mRNA expression by PMEG and PMEDAP. Initial MAPK expression profiling in CCRF-CEM lymphoblastic cells using 1 μM PMEG (approximate 50% growth inhibitory concentration at 72 h) revealed elevation of the *p38 MAPK* mRNA, whereas no changes were found in the mRNA for ERK and JNK kinase (Table I). Detailed expression analysis of all known *p38* isoforms (*α β, γ* and *δ*) in the two leukemia cell lines was then performed (Table II). It was found that the individual *p38* isoforms responded to PMEG treatment differently. *p38β* proved to be the most heavily up-regulated isoform, followed by *p38γ* in PMEG-treated CCRF-CEM cells. *p38δ* was not significantly altered and p38α was even down-regulated. HL-60 cells appeared to be less sensitive to PMEG treatment, demonstrating only minor changes in MAPK expression. The effect of PMEDAP on MAPK expression was not identical to that of PMEG (Figure 1B), despite their structural similarity. *p38β* was largely unaffected by PMEDAP, while most *MAPK* mRNAs tended to decrease. To gain a better idea of how individual *p38* isoforms contribute to the overall *p38* mRNA expression, we compared their basal mRNA levels. The most abundant *p38* isoforms in both cell lines tested were *δ* and *α* (Figure 1). The expression changes in these isoforms are thus most likely to influence total p38 MAPK protein level, which was found to be rather stable throughout PMEG and PMEDAP treatment (Figure 2, second row).

ERK and AKT pathways are activated in response to PMEG or PMEDAP. Phosphorylated (activated) forms of ERK, AKT, p38 and JNK were quantified using phospho-specific antibodies. Our findings indicate that in CCRF-CEM cells, both ERK and AKT pro-survival pathways were activated following PMEG and PMEDAP treatment. On the other hand, JNK and p38 did

Table II. *P38 MAPK mRNA expression in CCRF-CEM and HL-60 leukemia cells following their treatment with increasing concentrations of 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG) and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP). Data are means±SD of at least three independent experiments. *p<0.05 vs. untreated cells (ANOVA).*

	μM	CCRF-CEM				HL-60			
		p38α	*p38β*	*p38γ*	*p38δ*	*p38α*	*p38β*	*p38γ*	*p38δ*
PMEG	0.3	95±15	148±23	113±21	112±8	78±9	77±4	79±12	108±12
	1	69±3*	203±32	132±28	111±11	102±17	111±16	70±8	142±6
	3	38±15*	293±48*	212±12*	83±11	78±36	171±58	65±11*	156±21*
PMEDAP	1	95±14	154±31*	104±13	95±17	84±22	73±8	79±13	95±11
	3	81±4	114±20	109±27	92±11	73±19	90±14	58±20	121±20
	10	45±10*	96±15	74±4	67±7	44±24*	120±57	56±19	118±48

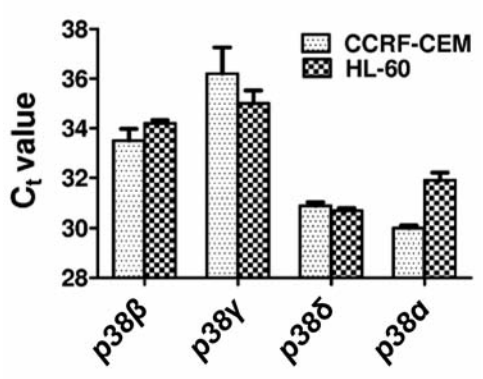

Figure 1. *Comparison of basal mRNA expression of the p38α, β, γ and δ isoforms in untreated CCRF-CEM and HL-60 cells. Data are expressed as C_t values which are negatively correlated to the respective mRNA transcripts present in a sample.*

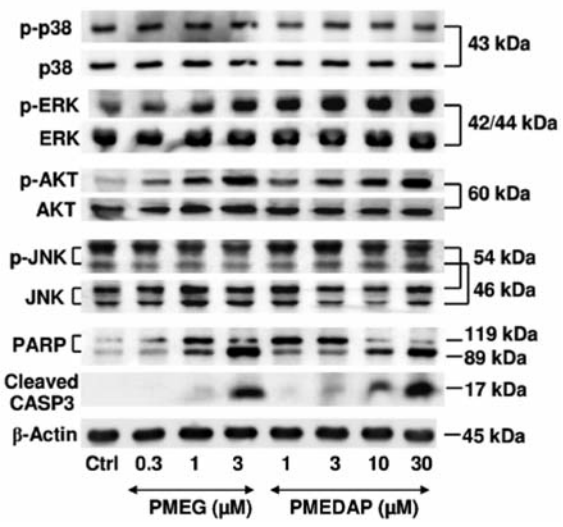

Figure 2. *Activation of MAPK and AKT kinase and cleavage of apoptotic hallmark proteins poly(ADP-ribose)polymerase (PARP) and caspase 3 (CASP3) in CCRF-CEM cells following 9-[2-(phosphonomethoxy)ethyl] guanine (PMEG) and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) treatment (72 h). Data are representative of two independent immunoblot analyses yielding similar results. β-Actin was used as a loading control.*

not appear to be activated (Figure 2). The MAPK phosphorylation pattern in HL-60 cells was very similar to the one observed in CCRF-CEM with an additional slight activation of p38 (not shown). The total protein level (phosphorylated and unphosphorylated) of all MAPKs under investigation remained unchanged following exposure to PMEG and PMEDAP.

Inhibition of p38 kinase prevents caspase 3-mediated apoptosis. In order to elucidate the role of individual MAPK pathways in PMEG- and PMEDAP-induced cytotoxicity and apoptosis cells were pretreated with 10 μM PD98059, SB203580, LY294002 and SP600125 representing selective inhibitors of ERK, p38, AKT and JNK, respectively. While there was no effect of pretreatment observed on cell proliferation/viability (Figure 3A and 3B), inhibition of p38 kinase clearly diminished both PMEG and PMEDAP-induced apoptosis as monitored by procaspase-3 cleavage (Figure 3C). On the other hand, inhibition of ERK potentiated the apoptotic response. JNK and AKT inhibitors had no effect (an apparent increase in cytotoxicity is due to the intrinsic toxicity of the inhibitors). The data indicate that p38 plays role in caspase-mediated apoptosis induced by the cytotoxic nucleoside phosphonates PMEG and PMEDAP.

ANPs are neither ERK nor AKT catalytic inhibitors. To determine whether PMEG, PMEDAP or structurally related compounds act as catalytic inhibitors of ERK or AKT kinases, a series of ANPs was screened in a direct kinase activity assay with human recombinant ERK or AKT (see Materials and Methods). Contrary to our expectations we found that none of the compounds tested acted as ERK or AKT inhibitors (Figure 4). Therefore, the cytotoxic properties (and other biological

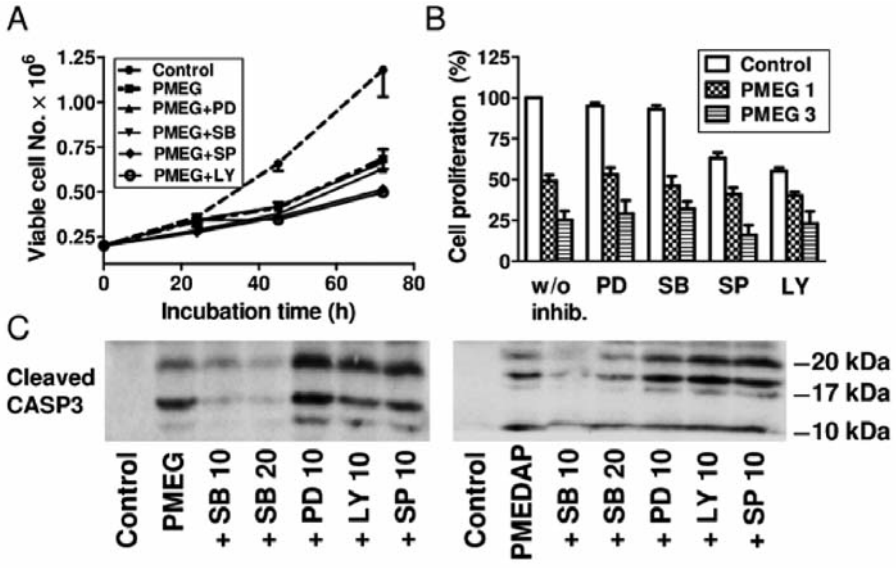

Figure 3. *Effects of selective MAPK inhibitors on 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG)-induced inhibition of cell proliferation and apoptosis. Panel A shows proliferation curves in the presence and absence of 1 μM PMEG and 10 μM inhibitors of ERK, p38, JNK or AKT kinase throughout a 72-hour incubation. Panel B demonstrates the intrinsic toxicity of inhibitors at 10 μM concentration and the influence of the inhibitors on antiproliferation activity of 1 μM or 3 μM PMEG. Diminution of PMEG- and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP)-induced apoptosis by p38 but not ERK, JNK or AKT inhibitor, as evidenced by CASP 3 cleavage (panel C). SB, SB203580 (p38 inhibitor); SP, SP600125 (JNK inhibitor); PD, PD98059 (ERK inhibitor); LY, LY294002 (AKT inhibitor).*

activities) of these compounds cannot be attributed to their ability to directly inhibit ERK or AKT kinases.

Discussion

MAPK signaling plays a crucial role in apoptosis and in cell survival. In this work we hypothesized that some MAPK members might play a role in the mode of cell death caused by PMEG and PMEDAP. p38 MAPK has been previously described to be involved in the apoptosis induced by gemcitabine (a pyrimidine nucleoside analogue) in pancreatic tumor cell lines (13), however, ANPs have not yet been investigated for this perspective. Since ANPs are generally more active against hematological malignancies compared to solid tumors, leukemia cells were chosen for this study. The primary mechanism of antitumor action of PMEG and PMEDAP is believed to be the incorporation of their diphosphates into DNA where they act as obligatory chain terminators and inhibit DNA polymerases (14). The role of apoptotic signaling has been recently addressed by our group (15), showing that PMEG-induced apoptosis involves caspase activation but is not strictly dependent on the caspase cascade. This appears to be in good agreement with the findings presented here. These demonstrate the key role of p38 MAPK in the caspase-mediated cell death induced by PMEG while at the same time caspase-independent pathways allow the compound to exert its cytotoxicity even under the conditions of complete p38 kinase inhibition.

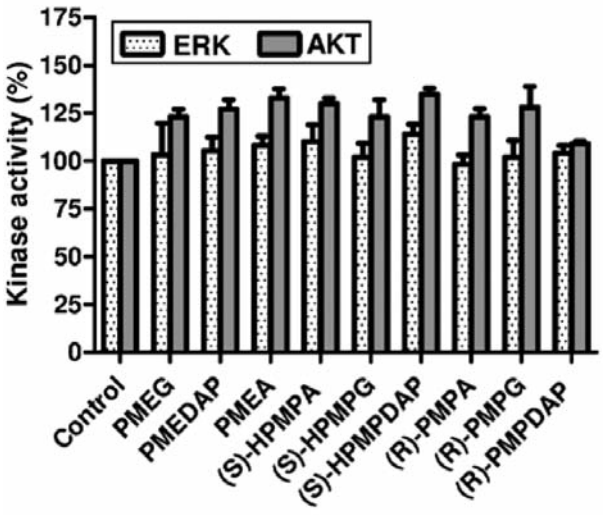

Figure 4. *In vitro inhibitory activity of a series of biologically active acyclic nucleoside phosphonates towards ERK2 and AKT kinases. Data are expressed as a percentage of reaction velocity of control (non-inhibited) sample±SD. PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; PMEDAP, 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; (S)-HPMPA, (S)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]adenine; (S)-HPMPG, (S)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]guanine; (S)-HPMPDAP, (S)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]-2,6-diaminopurine; (R)-PMPA, (R)-9-[2-(phosphonomethoxy)propyl]adenine; (R)-PMPG, (R)-9-[2-(phosphonomethoxy)propyl]guanine; (R)-PMPDAP, (R)-9-[2-(phosphonomethoxy) propyl]-2,6-diaminopurine.*

In this work, we have also found that although several *p38 MAPKs* were regulated by PMEG/PMEDAP at the mRNA expression level, this was not reflected in the total p38 protein level. This apparent inconsistency might be explained by the fact that the basal expression of the individual *p38 MAPK* isoforms differs by several orders of magnitude.

Since purine-derived nucleosides (5-iodotubercidin) have also been shown to act as ERK catalytic inhibitors (16) we investigated whether the biological effects of PMEG, PMEDAP or other ANPs might be linked with this inhibitory activity. Inhibition of AKT kinase, another kinase involved in pro-survival cell signaling, was also investigated. However, all the analogs tested failed to inhibit recombinant ERK or AKT in an *in vitro* assay.

Conclusion

In summary, we found that cytotoxic nucleotide analogs PMEG and PMEDAP modulate expression and activation of some MAPK signaling pathways molecules and that the cytotoxicity of these compounds is partly mediated by p38 kinase in human leukemia cells. However, involvement of p38 MAPK-independent pathways that omit the caspase cascade and directly activate PARP, clearly contribute to PMEG and PMEDAP-induced apoptosis. Antitumor effects of PMEG and PMEDAP do not involve ERK or AKT catalytic inhibition.

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