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Title page

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Abstract

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4 Acyclic nucleotide analogue PMEG represents promising drug candidate against lymphomas.
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7 In the present work we describe the ability of PMEG to induce resistance and we elucidate the
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9 mechanisms involved in this process. CCRF-CEM T-lymphoblastic cells resistant to either
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11 PMEG or its 6-amino congener PMEDAP were prepared and assayed for the expression of
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13 membrane transporters, PMEG and PMEDAP uptake and intracellular metabolism. Genes for
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15 guanylate kinase (GUK) and adenylate kinase (AK) isolated from PMEG- and PMEDAP-
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17 resistant cells were sequenced and cloned into mammalian expression vectors. PMEG-
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19 resistant cells were transfected with GUK vectors and catalytic activities of GUKs isolated
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21 from PMEG-sensitive and resistant cells were compared. PMEG phosphorylation to PMEG
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23 mono- and diphosphate was completely impaired in resistant cells. GUK obtained from
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25 PMEG-resistant cells revealed two point mutations S³⁵N V¹⁶⁸F that significantly suppressed
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27 its catalytic activity. Transfection of resistant cells with wtGUK led to the recovery of
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29 phosphorylating activity as well as sensitivity towards PMEG cytotoxicity. No differences in
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31 PMEG uptake have been found between sensitive and resistant cells. In contrast to GUK no
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33 changes in primary sequence of AK isolated from PMEDAP resistant cells were identified.
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35 Therefore, resistance induced by PMEDAP appears to be conferred by other mechanisms. In
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37 conclusion, we have identified GUK as the sole molecular target for the development of
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39 acquired resistance to the cytotoxic nucleotide PMEG. Therefore, PMEG is unlikely to cause
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41 cross-resistance in combination therapeutic protocols with most other commonly used
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43 anticancer drugs.
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56 **Keywords.** Resistance, acyclic nucleoside phosphonates, PMEG, PMEDAP, guanylate
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58 kinase, adenylate kinase.
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1 **Abbreviations.** PMEG, 9-[2-(phosphonomethoxyethyl)guanine]; PMEDAP, 9-[2-
2 (phosphonomethoxyethyl)diaminopurine]; PMEA, 9-[2-(phosphonomethoxyethyl)adenine];
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4 GUK, guanylate kinase; AK, adenylate kinase; GMP, guanosine monophosphate; AMP,
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6 adenosine monophosphate; ANP, acyclic nucleoside phosphonate; CdA, 2-chloro-2'-
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8 deoxyadenosine (cladribine); FUDR, 5-fluoro-2'-deoxyuridine
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1. Introduction

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2 Nucleoside and nucleotide analogs rank among clinically important drugs in
3 anticancer chemotherapy. Although acyclic nucleoside phosphonates (ANP) have now been
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7 predominantly recognized as efficient antiviral agents [1], their anticancer potency is also of
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10 interest [2]. 9-[2-(phosphonmethoxyethyl)guanine (PMEG) and 9-[2-
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12 (phosphonmethoxyethyl)diaminopurine (PMEDAP) (Fig. 1) represent the ANP with
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14 enhanced cytotoxic properties and possible use as novel antitumor compounds [3,4]. PMEG,
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16 in a form of a double prodrug GS-9219, has been previously shown to be active against Non-
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18 Hodgkin's lymphoma in dogs [5], while PMEDAP significantly prolonged survival of SD-rats
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20 with spontaneous lymphoma [6]. The advantage of ANP over conventional nucleotides lies in
21
22 the chemical and metabolic stability of phosphonic bond. ANP bypass first-step
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24 phosphorylation, nevertheless they still require phosphorylation to ANPp and ANPpp to be
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27 active [7]. These reactions are catalyzed by specific nucleoside monophosphate kinases
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29 (NMPK) and relatively non-specific nucleoside diphosphate kinase (NDPK). We have
30
31 previously demonstrated that PMEG is phosphorylated by guanosine monophosphate kinase
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33 (guanylate kinase, GUK) [8] while PMEDAP is activated by the mitochondrial isoform of
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35 adenosine monophosphate kinase (adenylate kinase 2, AK2) but not its cytosolic AK1
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37 counterpart in L1210 cells [9]. These studies also indicated that both PMEG and PMEDAP
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39 were much weaker substrates for their respective phosphorylating enzymes compared to the
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41 natural substrates GMP and AMP, respectively.
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48 Acquired resistance to chemotherapy upon prolonged or repeated administration is a
49
50 serious issue complicating the treatment. Understanding the mechanisms leading to its
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52 development is crucial for designing strategies how to prevent or delay its onset as well as for
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54 predicting possible cross-resistance with other chemotherapeutics used within the same
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therapeutical protocol. Frequently, resistance occurs as a consequence of decreased

1 intracellular concentration of the drug due to up-regulation of drug efflux proteins such ATP-
2 binding cassette transporters (P-gp, MRP1, MRP4, MRP5) [10]. While nucleotides are not
3
4 recognized as P-gp substrates, there is some evidence that MRP4 and/or MRP5 transporters
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6 may play role in resistance to 9-[2-(phosphonomethoxyethyl)adenine (PMEA) [11] and
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8 PMEDAP [12]. Another possible cause of the resistance that has been described in some
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10 anticancer nucleosides is represented by the defective metabolic activation, i.e.
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12 phosphorylation, by the individual kinases [13] or enhanced deactivation by nucleotidases
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14 [14]. Other means of chemoresistance development include alterations in various signaling
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16 pathways such as protein kinase signaling [15].
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22 This work aims to elucidate the mechanisms responsible for the development of
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24 resistance in CCRF-CEM lymphoblastic cells following long-term exposure to PMEG.
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26 Emphasis has been placed on the role of intracellular transport and metabolism. The question
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28 whether there might be interferences with the cytostatic efficiency of other commonly used
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30 chemotherapeutics has also been addressed.
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41 **2. Methods**

42 *2.1. Materials*

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46 PMEG and PMEDAP were prepared according to the previously published procedures [16].
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49 The identity and purity of the compounds was verified by means of NMR spectroscopy. Stock
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51 solutions of the compounds were prepared by dissolving them in water to 15 mM
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53 concentration. Doxorubicin, etoposide, cladribine, 5-fluoro-2'-deoxyuridine, 1,1,2-trichloro-
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56 1,2,2-trifluoroethane, trioctylamine, mineral oil, silicone oil DC 702, streptomycin, penicillin
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1 G, PBS and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA),
2 fetal calf serum was obtained from PAA Laboratories GmbH (Pasching, Austria). [8-
3 ³H]PMEG and [8-³H]PMEDAP were prepared at the Laboratory of Radioisotopes at IOCB
4 [17], [U-¹⁴C]GMP and [8-¹⁴C]AMP were purchased from MP Biomedicals (Solon, OH,
5 USA). Soluene 350[®] was provided by Perkin Elmer (Waltham, MA, USA), TCA and salts for
6 buffer preparations were from Serva (Heidelberg, Germany), LY335979 and MK571 were
7 kindly provided by Gilead Sciences (Foster City, CA, USA). Oligonucleotides (PCR primers)
8 used in this study were custom-synthesized by Sigma-Aldrich.
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22 2.2. Cell culture

23 CCRF-CEM cells (ATCC CCL 119) were cultured under a humidified atmosphere containing
24 5% CO₂ at 37 °C. They were grown in T-25 flasks in RPMI-1640 medium supplemented with
25 10% (v/v) fetal calf serum, 200 µg.mL⁻¹ of streptomycin, 200 U.mL⁻¹ of penicillin G and 4
26 mM glutamine. Resistant cells were obtained by continuous exposure of the cells to
27 increasing concentrations of PMEG or PMEDAP starting at their respective GIC₅₀ (1 µM and
28 10 µM), and reaching 90 µM and 300 µM, resp. after 12 months. Cells were subcultured
29 twice a week by centrifugation and fresh media with the compounds were added each time.
30 Cell growth and viability was monitored using Countess[®] Automated Cell Counter
31 (Invitrogen, Paisley, UK) following Trypan blue (0.4%) staining.
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49 2.3. Cytotoxicity evaluation

50 Sensitivity of the cells to various chemotherapeutics (PMEG, PMEDAP, doxorubicin,
51 etoposide, cladribine, FUDR) was assessed with the use of XTT cell proliferation kit II
52 (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions.
53 Briefly, cells were seeded in a 96-well plate in a density of 10 000 cells per well and left to
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1 rest o/n. The tested compounds were added to the culture media the next day and incubated
2 for 72 h before XTT dye was added. The absorbance at 495 nm was read after 1 h. IC₅₀ values
3 were determined by GraphPad Prism version 5.00 for Windows (GraphPad Software, La
4 Jolla, CA, USA).
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10 2.4. Intracellular transport of [³H]-PMEG and [³H]-PMEDAP

11 CCRF-CEM cells were washed by centrifugation (250 x g, 5 min) in PBS and resuspended in
12 RPMI medium w/o any additives. The cell suspension was distributed into microtubes in 450-
13 µl aliquots and 50 µl [³H]PMEG or [³H]PMEDAP was added to the desired concentration.
14 Incubation was done at 37 °C in a controlled CO₂-incubator using a rotary stirrer. At indicated
15 time intervals the uptake process was terminated by centrifugation at 5300 x g for 1 min
16 through an oil layer (a mixture of silicone and mineral oil at final specific density of 1.05 g/ml
17 of 150 µl volume). The cell sediment was washed by centrifugation (5300 x g, 1 min) in 1 ml
18 PBS, solubilized with Soluene[®] tissue solubilizer o/n and radioactivity was counted in a
19 toluene scintillator (4 ml per sample). The intracellular volume of CCRF-CEM for calculation
20 of the actual cytoplasmic concentration of PMEG and PMEDAP was 3.38 ml/10⁷ cells as
21 determined previously (Olšanská et al., 1997).
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43 2.5. Intracellular metabolism of [³H]PMEG and [³H]PMEDAP in sensitive and resistant cells

44 The cells were washed with PBS, resuspended in 20 ml of RPMI growth medium at a
45 concentration of 1 x 10⁶ per ml and incubated with 2.5 µM [³H]PMEG (200 µCi) or 15 µM
46 [³H]PMEDAP (200 µCi) in a CO₂-incubator for 24 h at 37 °C. Cells were then washed in 1 ml
47 PBS and pelleted by centrifuging at 250 x g for 1 min. The sediment was resuspended in 200
48 µl of deionized water and subjected to three freeze-thaw cycles. 200 µl of 10% trichloroacetic
49 acid (TCA) was then added. After 10 min of vigorous shaking at 4 °C the precipitate was
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1 sedimented at 11000 x g (5 min). TCA was extracted from the supernatant with 400 µl of a
2 mixture of 1,1,2-trichloro-1,2,2-trifluoroethane–trioctylamine (4 : 1, v/v). The two phases
3 were separated by centrifugation at 11000 x g for 5 min and 50 µl of the upper aqueous layer
4 was applied to Supelcosil LC-18T HPLC column (150 x 4.6 mm, 3 µm) and analyzed for
5 PMEG and PMEDAP metabolites. Elution buffer C contained 50 mM KH₂PO₄ and 3 mM
6 tetrabutylammonium hydrogensulphate at pH 3.1. Buffer D was identical with C except for
7 the addition of 30% (v/v) acetonitrile. The column was eluted with a linear gradient from 15
8 to 60% of buffer D for 25 min at the flow rate of 1 ml/min. 0.5-ml fractions were collected
9 and radioactivity was counted in an aqueous scintillator (4 ml per sample). PMEG, PMEGp,
10 PMEGpp, PMEDAP, PMEDAPp and PMEDAPpp were identified with the aid of authentic
11 standards.
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29 2.6. Preparation of crude cellular extracts and *in vitro* phosphorylation studies

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31 1.5 x 10⁹ cells were washed with PBS and suspended in 1.5 ml of 50 mM Tris-HCl buffer pH
32 7.4 containing 1 mM DTT, 5 mM MgCl₂, protease inhibitor cocktail (Sigma-Aldrich) and
33 0.4% NP-40. Cells were sonicated 3 x 5 s and homogenized in a Dounce homogenizer.
34 Homogenate was centrifuged at 30000 x g for 30 min. Supernatant was incubated with
35 streptomycin sulphate for 1 h to remove nucleic acids and centrifuged at 30000 x g for 30
36 min. Resulting crude cell extract (supernatant) was desalted on PD-10 columns (GE
37 Healthcare), aliquoted and stored at -80 °C. Protein content was determined by the BCA kit
38 (Sigma-Aldrich), according to the manufacturer's instructions. Reaction mixture (20 µl) for *in*
39 *vitro* phosphorylation of PMEG or guanosine monophosphate (GMP) contained 100 mM
40 TRIS-HCl buffer pH 7.5, 4 mM ATP, 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 5 µCi
41 [³H]PMEG or 0.05 µCi [¹⁴C]GMP, varying concentration of cold PMEG or GMP and 10 µl of
42 cell extract (7-9 mg/ml protein for PMEG phosphorylation, 0.1 mg/ml protein for GMP
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1 phosphorylation). Reaction mixture (20 μ L) for *in vitro* phosphorylation of PMEDAP or
2 adenosine monophosphate (AMP) contained 40 mM HEPES buffer pH 8.0, 1 mM $MgCl_2$, 0.2
3 mM ATP, 2 mM mercaptoethanol, 0.4 mg/ml BSA, 5 mM creatine phosphate, 0.05 mg/ml
4 creatine kinase, 5 μ Ci [3H]PMEDAP or 0.05 μ Ci [^{14}C]AMP, varying concentration of cold
5 PMEDAP or AMP and 10 μ l of cell extract (7-9 mg/ml for PMEDAP phosphorylation,
6 diluted to 0.01 mg/ml protein for AMP phosphorylation). The reactions took place at 30 $^{\circ}C$
7 for 10 min and were terminated at indicated time by spotting a 2 μ l aliquot onto a PEI-
8 cellulose TLC plate prespotted with authentic standards and developed in 0.5 M KH_2PO_4 . The
9 spots were visualized under UV light and radioactivity was counted in a scintillation counter.
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24 2.7. Cloning and sequencing of GUK and AK genes

25 RNA isolation, quality control and cDNA synthesis were performed as described previously
26 [18]. cDNA was prepared from total mRNAs isolated from PMEG sensitive or resistant
27 CCRF-CEM cells, and PMEDAP sensitive or resistant CCRF-CEM cells. The GUK1 gene
28 was isolated from PMEG sensitive and resistant cells using GUK1 specific primers, 5'AAA
29 GAA TTC GGA TGT CGG GCC CCA GGC CTG TG and 3'AAA CTC GAG TCA GGC
30 GCC GGT CCT TTG AGC. AK2 A and B isoforms were isolated from PMEDAP sensitive
31 and resistant cells using the same primer at the 5'end 5'AAA TGG CCA TGG CTC CCA
32 GCG TGC CAG C and 3'AK2 specific primers: AAA CTC GAG TTA GAT AAA CAT
33 AAC CAA GTC TTT AC for AK2A and AAA CTC GAG CTA GGA TGT GGC TTT GGA
34 GAA GG for AK2B, respectively. PCR was carried out using Phusion polymerase
35 (Finnzymes, Espoo, Finland). The PCR products were cloned into HA-pCMV vector
36 (Clontech, Mountain View, CA, USA). Five independent clones of each isolate were
37 sequenced. The amino acid alignment was done using Multalin software at
38 <http://multalin.toulouse.inra.fr/multalin/multalin.html>.
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2.8. Transfection of the cells with wt and SV/NF GUK DNA

2 ml of RPMI medium w/o antibiotics, containing 10% FCS were placed into each well of the 6-well plate and left to equilibrate at 37 °C in a CO₂ incubator for 2-3 hours. Sensitive and PMEG-resistant cells were harvested by centrifugation (250 x g, 5 min), washed with PBS and suspended in Resuspension buffer R (Neon[®] 10 µL kit, Invitrogen) containing 0.1 µg/µl DNA or PBS (mock) to a concentration of 200 000 cells/µl. 10 µl of cell suspension were aspirated into a Neon[®] tip and subjected to three pulses of 1325 V for 10 ms using Neon[®] Transfection System (Invitrogen). Transfected cells were transferred in the prewarmed medium in the culture plate and incubated for 48 h. After that, cells were lysed either in the protein lysis buffer for immunoblotting experiments or in the CHAPS lysis buffer for the assays of enzymatic activity. 30000 x g supernatants were immediately used for determination of protein content and GUK and AK activity as described in 2.6.

2.9. RT-qPCR analysis of gene expression

GUK1, AK2 and β-actin mRNA expression was quantified using DyNamo[®] kit (Finzymmes) and DNA Engine Opticon[®] 2 thermocycler (BioRad, Herkules, CA, USA). The PCR reactions (25 µl) were run in triplicates and contained 2 µl of template cDNA and 0.3 µM of the following primers: GUK1(FP) – CTC CTC TGT GGC TCT GGA AG; GUK1(RP) - CTC TTC AGC AGG GTG CTC TT; AK2(FP) - TAT CCT AAA GGC ATC CGG G; AK2(RP) - CCC CAG TAG CTA AAT GGC AG. The thermocycling program included an initial denaturation at 95 °C for 15 min and 40 cycles of 95 °C for 35 s, 56 °C for 30 s and 72 °C for 30 s. The specificity of the amplified product was verified by melting curve analysis. Expression levels of target genes were normalized to β-actin. Drug transporters' expression

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2 profiling was performed using RT²-Profiler PCR arrays (SABiosciences, Frederick, MD,
3 USA) according to manufacturer's instructions.
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7 *2.10. Immunoblotting*

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9 10⁷ cells were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors
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11 (Pierce, Rockford, IL). 30 µg of total protein were loaded on 12% polyacrylamide gel,
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13 electrophoresed and electroblotted onto a PVDF membrane. Membranes were then blocked in
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15 5% non-fat dry milk (Cell Signaling Technology (CST), Danvers, MA) in TBS containing
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17 0.05% of Tween 20 (Pierce) and probed with anti-GUK or anti-AK2 antibody (Sigma-
18
19 Aldrich) and appropriate HRP-conjugated secondary antibody (CST). SuperSignal[®] West
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21 Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce) was used for signal
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23 detection by CCD camera. The membrane was then stripped with Restore[™] Western Blotting
24
25 Stripping Buffer (Pierce) according to manufacturer's instructions and reprobed with anti-β-
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27 actin antibody (CST) as a loading control. Monoclonal anti-HA peroxidase-conjugated
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29 antibody (Sigma) was used to verify efficiency of transient expression of HA-GUK.
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39 *2.11. Data analysis and statistical procedures*

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41 Unless otherwise indicated the data are presented as mean ± S.D. from at least three
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43 independent experiments. Statistical evaluation was performed using GraphPad Prism version
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45 5.00 for Windows (GraphPad Software).
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3. Results

3.1. PMEG and PMEDAP uptake is not modified in resistant cells

To determine the role of the transport in the resistance to PMEG and PMEDAP, we first screened the cells for the mRNA expression of various drug transporter proteins. Several genes were upregulated in resistant cell including P-gp (MDR1, ABCB1), whose expression was 5-fold higher in PMEG-resistant cells and 19-fold higher in PMEDAP-resistant cells compared to control, thus representing the greatest mRNA increase of all tested genes (Table 1). Since nucleotides are not recognized as P-gp substrates, we have checked for the effect of two specific and efficient P-gp inhibitors (LY335979, XR9576) on the sensitivity of resistant cells towards PMEG and PMEDAP. It turned out that P-gp inhibition by XR9576 did not enhance cytotoxicity of any of the tested compounds (Table 2). Experiments with LY335979 gave identical results (data not shown). These findings indicate that PMEG- and PMEDAP-induced resistance is most likely P-gp independent. This is further supported by the observation that both resistant cell lines retained sensitivity towards doxorubicin and etoposide, traditional P-gp substrates (Fig. 2A). On the other hand, MK571, a selective inhibitor of another class of resistance proteins known as MRPs, slightly reversed resistance of PMEDAP-resistant cells to PMEG (3-fold decrease in IC_{50}) and possibly also to PMEDAP. However, IC_{50} for PMEDAP in resistant cells was not reached for up to 300 μ M. Sensitivity of PMEG-resistant cells to either PMEG or PMEDAP remained unaffected by MK571. Interestingly, we also observed that the cross-resistance between PMEG and PMEDAP is not strictly reciprocal, i.e. PMEG-resistant cells are highly resistant to both PMEG and PMEDAP ($IC_{50} > 300 \mu$ M), whereas PMEDAP-resistant cells retain certain degree of sensitivity to PMEG ($IC_{50} = 49 \mu$ M) while it is highly resistant to PMEDAP ($IC_{50} > 300 \mu$ M) (Table 2). This strongly suggests that in spite of the close structural resemblance of the two compounds

1 (a single amino group difference) the molecular target responsible for the development of
2 resistance may not be identical. Finally, we performed direct tracking of PMEG and
3
4 PMEDAP in sensitive and resistant cells using radiolabeled compounds. As depicted in Figure
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7 3, no significant variations in the intracellular concentration of PMEG or PMEDAP were
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9 found between sensitive and resistant cells for up to 45 min (Fig. 3A,C), irrespectively of the
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11 concentration used (Fig. 3B,D). It should be noted that this assay reflects total intracellular
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13 concentration of both the parent compounds and their metabolites.
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19 3.2. PMEG and PMEDAP are not phosphorylated in resistant cells

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21 After excluding a major role of diminished drug uptake, intracellular phosphorylation of
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23 PMEG and PMEDAP was investigated. Incubation of the cells with 2.5 μM [^3H]PMEG or 15
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25 μM [^3H]PMEDAP for 24 h resulted in a complete absence of PMEGp and PMEGpp in
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27 PMEG-resistant cells (Fig. 4B) as well as PMEDApp and PMEDAPpp in PMEDAP-resistant
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29 cells (Fig. 4D). All metabolites were detectable in sensitive cells (Fig. 4A,C). These data
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31 indicate a block at the level of nucleoside monophosphate kinases. Further *in vitro*
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33 experiments with crude cellular extracts confirmed the incapacity of PMEG-resistant cells to
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35 phosphorylate PMEG as well as GMP (Table 3), clearly pointing to GUK to be responsible
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37 for the resistance. On the other hand, phosphorylation of PMEG was largely preserved in
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39 PMEDAP-resistant cells. Unfortunately, PMEDAP metabolites could not be detected in this
40
41 cell-free assay. However, the metabolism of AMP (a natural substrate for AK) demonstrated
42
43 that phosphorylation rate was slightly depressed in PMEDAP-resistant cells while it remained
44
45 unaffected in PMEG-resistant cells (Table 3). Resistance to PMEDAP therefore appears to be
46
47 dependent on AK although this effect is much weaker compared to PMEG vs. GUK.
48
49 Impairment of AK in PMEDAP-resistant cells is supported by the observation that cladribine,
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51 a nucleotide analogue that also uses AK to its metabolic activation, was 10 times less
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cytotoxic in PMEDAP-resistant cells compared to sensitive or PMEG-resistant cells. On the other hand, the cytotoxicity of 5-fluoro-2'-deoxyuridine (FUDR), an analogue that requires neither GUK nor AK to its activation, was not affected in any of the two resistant cell lines (Fig. 2B).

3.3. Expression of GUK and AK2 is decreased in resistant cells

To determine whether the lack of phosphorylating capacity is due to decreased expression of the respective activating kinases (GUK for PMEG, AK2 for PMEDAP) or rather their defective catalytic activity, mRNA and protein levels of GUK and AK2 were quantified. We found an approx. 40% drop in GUK mRNA expression in both resistant cell lines whereas AK2 expression was only decreased in PMEDAP-resistant cells (50% of control level) (Fig. 5A). Protein levels appeared to be influenced in a similar manner although GUK protein was poorly detectable by immunoblotting due to its relatively low abundance compared to AK2 (Fig. 5B).

3.4. Catalytic activity of GUK from PMEG-resistant cells is impaired due to point mutations

To determine possible mutations leading to changes in catalytic activities, the genes encoding both GUK and AK2 were isolated from the sensitive and resistant cells, cloned into mammalian expression vector and sequenced. No changes in primary sequences of both A and B AK2 isoforms isolated from PMEDAP resistant and sensitive cells were found. In contrast, GUK isolated from PMEG-resistant cells revealed two point mutations S³⁵N and V¹⁶⁸F (SV/NF GUK) (Fig. 6). According to the 3-D structure of highly similar mouse GUK, sharing 93% homology with human GUK, it is likely that both identified mutations affect the CORE domain of GUK (Sekulic et al., 2002). The S³⁵ lies within the hinge 1 connecting

1 NMP-binding and CORE regions and V¹⁶⁸ lies in helix 7 within the CORE domain. As both
2 residues S³⁵ and V¹⁶⁸ are highly conserved among GUK genes we hypothesized that at least
3
4 one of these mutations negatively affects catalytic activity of GUK thus conferring PMEG
5
6 resistance. To verify this, the PMEG resistant cells were transfected with the wtGUK and
7
8 SV/NF GUK expression vectors. The levels of expression of both wt and SV/NF GUK
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10 proteins in transfected cells were determined by immunoblotting using antibody against HA
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12 (Fig. 7A) and anti-GUK (Fig. 7B). We observed a 20-fold increase in GMP phosphorylating
13
14 activity and 33-fold increase in PMEG phosphorylating activity in PMEG-resistant cells
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16 transfected with wtGUK DNA whereas the cells transfected with the SV/NF GUK showed
17
18 only a minor increase (2.5-fold for GMP and no change for PMEG as a substrate) (Table 4).
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20 In addition to the re-established cellular phosphorylation activity, transfection of resistant
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22 cells with wtGUK DNA restored the sensitivity to the cytotoxic effects of PMEG, which is
23
24 evidenced by the drop of IC₅₀ for PMEG from more than 300 μM to 12 μM (Table 4).
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39 4. Discussion

41 This work elucidates the mechanisms leading to the resistance of T-lymphoblastic
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43 cells to the cytotoxic acyclic nucleotide PMEG, which has potential as new antilymphoma
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45 drug. Besides PMEG, its close structural congener PMEDAP was also investigated for
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47 comparative purposes. Moreover, PMEDAP-induced resistance was already addressed in the
48
49 work of Zápotocký et al. [12] indicating that MRP4 and MRP5 may be involved in the
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51 development of resistance in CCRF-CEM cells and in PMEDAP-treated SD/Cub rats. In that
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53 particular study, the resistance was induced by continuous exposure of the cells and the
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55 animals to a fixed concentration of PMEDAP (corresponding to the initial IC₅₀) for the whole
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selection period. Contrary to that we have now prepared cells resistant to much higher concentrations of PMEG and PMEDAP, respectively (40-100x initial IC_{50}). Under the conditions of our study, no changes in MRP4 or MRP5 mRNA were found in either PMEG- or PMEDAP-resistant cells. This apparent discrepancy can signify that the changes in MRP expression are more important at early phases of resistance development whereas other mechanisms predominate later on. Despite the lack of MRP mRNA upregulation, our experiments with MRP inhibitor MK-571 indicated that MRP transporters indeed affect PMEDAP cytotoxicity in PMEDAP-resistant cells. This can be possibly explained by the alterations in MRP function. On the other hand, PMEG-resistant cells were found to be fully independent on MRP proteins. The role of MDR1 protein (P-gp) was also ruled out by means of specific P-gp inhibitors, in spite of the fact that the initial expression analysis showed significant and puzzling increase in P-gp mRNA in both resistant cell lines. Nevertheless, P-gp mRNA overexpression did not result in the development of multidrug-resistance phenotype as documented by sustained sensitivity of the cells to the typical P-gp substrates (doxorubicin, etoposide) [19]. Direct measurements of intracellular concentrations of PMEG confirmed the assumption that the resistance is not due to changes of drug uptake. In case of PMEDAP, approximately 4-fold decrease in intracellular levels of PMEDAP was detected in PMEDAP-resistant cells compared to sensitive cells in a 24 h-experiment designed primarily to study the intracellular metabolism. Interestingly, this effect was not observed in a short term transport study (for up to 45 min) suggesting that PMEDAP enters PMEDAP-resistant cells rather efficiently but it is slowly expelled from the cells at later time points. This would be consistent with the above discussed role of MRP transporters in this process, however, more experiments would be necessary to confirm this hypothesis.

Possible defects in the metabolic activation of PMEG and PMEDAP to the corresponding diphosphates (dNTP analogues) were also investigated. It is known that

1 resistance to nucleoside analogs such as for example cladribine often results from decreased
2 levels of phosphorylated forms. This can be due to decreased activity of phosphorylating
3 kinases and/or increased activity of 5'-nucleotidases (5-NT) [13]. We have found that PMEG
4 and PMEDAP mono- and diphosphates were absent as well. The eventuality of enhanced
5 dephosphorylation can be ruled out since the phosphate-mimicking phosphonic group of
6 PMEG and PMEDAP resists hydrolysis by 5-NTs. We have therefore focused on
7 phosphorylating kinases that have been previously described in our group to catalyze PMEGp
8 and PMEDAPp formation – GUK and AK2, resp. [8,9]. The fact that each of the two
9 compounds uses a different enzyme for their first-step phosphorylation would give grounds
10 for the absence of a complete cross-resistance between each other. Expression of the two
11 kinases was somewhat depressed in resistant cells but not to the extent that would explain a
12 complete loss of phosphorylating capacity. Therefore, we assumed there might be some
13 alterations in the catalytic activity of these proteins possibly due to changes in primary
14 sequence, abnormal posttranslational modifications or other mechanisms. Upon isolation and
15 sequencing of GUK gene from PMEG-resistant cells, we identified two point mutations S³⁵N
16 and V¹⁶⁸F compared to GUK isolated from the PMEG-sensitive cells. As no high-resolution
17 structure of human GUK is available so far, we used the crystallographic structure of mouse
18 GUK to localize S³⁵N and V¹⁶⁸F mutations within the tertiary structure of the enzyme. It is
19 believed that due to high sequence similarity between mouse and human GUKs (88% identity,
20 93% homology) the structural information gained on the mouse GUK is directly transferable
21 to the human enzyme [20,21]. Assuming that this hypothesis is correct, the S³⁵ residue occurs
22 in the hinge connecting the helix 1 of CORE region and the helix 2 of NMP-binding region.
23 S³⁵ is highly conserved within the primary structure of GUK and does not change from
24 bacteria to human. The V¹⁶⁸F mutation occurs within the helix 7 of the CORE region and this
25 residue is replaced with isoleucine in porcine or bovine GUKs or leucine in bacterial GUK.
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Interestingly, a mutation in a close proximity of S³⁵ of mouse GUK has been proposed to be responsible for resistance to 6-thioguanine [21]. In that work Ardiani et al. substituted serine 37 and three resulting mutants S³⁷A, S³⁷T and S³⁷Y phosphorylated GMP but not 6-TG. We isolated GUK double mutant directly from the PMEG resistant cells and proved that the S³⁵NV¹⁶⁸F GUK is inactive not only towards PMEG but also GMP. These mutations in human GUK explained the mechanism of PMEG resistance. On the other hand, PMEDAP-phosphorylating kinase AK2 possessed wild-type genotype in both PMEG- and PMEDAP-resistant CCRF-CEM cells. Therefore, no changes in the conformation of AK2 protein and its catalytic activity can be anticipated and resistance to PMEDAP is clearly conferred by the mechanisms distinct from mutations in the activating kinase. Whether a 50% decrease in the AK2 protein content can itself explain the absence PMEDAP metabolism remains speculative. However, PMEDAP is only a poor substrate for AK2 (K_m = 670 μM) [9], which is probably the main reason why PMEDAP phosphorylation could not be detected in cell-free extracts. Therefore, PMEDAP metabolism could be more affected by the decreased expression of AK compared to PMEG vs. GUK. In addition, diminished intracellular concentration of PMEDAP in PMEDAP-resistant cells (Fig. 4) further lowers the amount of the substrate available for AK.

In conclusion, we have shown here that the potential anticancer drugs PMEG and PMEDAP are capable of inducing resistance upon prolonged treatment. PMEG-induced resistance is not due to the modifications in its uptake/efflux but solely due to a decreased capacity of resistant cells to metabolically activate (phosphorylate) the drug. This is largely conferred by the impaired catalytic function of GUK resulting from two point mutations and to a much lesser extent by the down-regulation of GUK. Resistance to PMEDAP is clearly distinct from that of PMEG and do not involve changes in the primary structure of the

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activating kinase. We have also shown that PMEG- and PMEDAP-induced resistance is not that of multidrug-resistance phenotype and is unlikely to affect other commonly used anticancer drugs. These findings have implications in clinical settings.

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7. Tables

Table 1. Gene expression profiling on drug transporters* in PMEG- and PMEDAP-resistant cells. The table shows the list of genes that have been upregulated in at least one of the two resistant cell lines compared to sensitive CCRF-CEM cells.

Symbol	Description	Gene name	Fold up-regulation	
			PMEG-res	PMEDAP-res
ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2	ABC2	3.5	4.4
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABC20/CD243	4.7	19.4
MVP	Major vault protein	LRP/VAULT1	2.6	2.3
SLC5A4	Solute carrier family 5 (low affinity glucose cotransporter), member 4	SAAT1/SGLT3	2.1	1.1
ABCB6	ATP-binding cassette, sub-family B (MDR/TAP), member 6	ABC/ABC14	1.0	2.0
ABCD1	ATP-binding cassette, sub-family D (ALD), member 1	ABC42/ALD	1.8	2.2
ATP6V0C	ATPase, H ⁺ transporting, lysosomal 16kDa, V0 subunit c	ATP6C/ATP6L	1.3	2.0
SLC15A2	Solute carrier family 15 (H ⁺ /peptide transporter), member 2	PEPT2	1.1	3.6
SLCO2A1	Solute carrier organic anion transporter family, member 2A1	OATP2A1/PGT	1.8	3.6
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	ABC17/ABCB2	1.1	2.1

*RT² Profiler™ PCR Array Human Drug Transporters (PAHS-070D, SABiosciences) enabling the analysis of mRNA for 84 transporter genes.

Table 2. Sensitivity of resistant cells to PMEG and PMEDAP and the influence of MDR and MRP inhibitors. Data are expressed as IC₅₀ (μM) obtained from XTT cytotoxicity test and represent means from three independent experiments.

Cell line	PMEG			PMEDAP		
	-	+XR	+MK	-	+XR	+MK
Control (WT)	2.7±0.2	3.3±0.3	3.5±0.6	19.0±2.8	31.0±9.1	19.4±0.4
PMEG-res	>300	>300	>300	>300	>300	>300
PMEDAP-res	49.0±7.1	49.3±6.9	17.8±0.8**	>300	>300	>300

XR – XR9576 (P-gp inhibitor), MK – MK576 (MRP inhibitor), **p<0.01 vs. resistant cells w/o inhibitor (ANOVA)

Table 3. *In vitro* phosphorylating capacity of crude cellular extracts from sensitive and resistant CCRF-CEM cells. Data are means from three independent experiments.

Cell line	PMEG		Substrate GMP		AMP	
	K_m^a	V_{max}^b	K_m	V_{max}	K_m	V_{max}
Control (WT)	140±17	0.17±0.01	15±3	50±3	6±3	355±45
PMEG-res	1253±195**	0.033±0.01**	270±151*	3±2**	7±3	424±37
PMEDAP-res	104±15	0.11±0.01**	8±2	33±2**	3±4	219±51*

^a[μ M]; ^b[$\text{nmol}^{-1}\text{min}^{-1}\text{mg}^{-1}$], * $p < 0.05$, ** $p < 0.01$ vs. control (ANOVA)

Table 4. Catalytic activity of PMEG-resistant CCRF-CEM cells transfected with wtGUK and SV/NF GUK and their sensitivity to the cytotoxicity of PMEG.

	GMP _v (nmol ⁻¹ min ⁻¹ mg ⁻¹) ^a	PMEG _v (nmol ⁻¹ min ⁻¹ mg ⁻¹) ^b	PMEG IC ₅₀ (μM) ^c
PMEG-res + wtGUK	66.5±0.5 ^{***}	4.55±0.40 ^{***}	12.6
PMEG-res + SV/NF GUK	8.4±1.1 ^{***}	0.13±0.00	>300
PMEG-res mock	3.3±1.0	0.14±0.02	>300
sensitive (wt) cells	19.6±0.6	0.39±0.02	1

*** p<0.001 vs. mock-transfected cells (One-Way ANOVA with Tukey's post-hoc test);

^a100 μM GMP, 30° C, 5 min; ^b100 μM PMEG, 30°C, 10 min; ^cXTT cytotoxicity test, 72 h post-transfection.

8. Figure captions

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5 Fig. 1. Structures of 9-[2-(phosphonmethoxyethyl)guanine] (PMEG) and 9-[2-
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7 (phosphonmethoxyethyl)diaminopurine] (PMEDAP).
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11 Fig. 2. Sensitivity of resistant cells to P-gp substrates (A) and other nucleoside analogs (B).

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13 Data are expressed as IC₅₀ values (nM) obtained from XTT cytotoxicity test and represent
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15 means from three independent experiments. FUDR - 5-fluoro-2'-deoxyuridine. ***p<0.01 vs.
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17 sensitive cells (ANOVA).
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25 Fig. 3. Uptake of [³H]PMEG and [³H]PMEDAP in sensitive and resistant CCRF-CEM cells.

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27 The time course of the intracellular transport was measured for up to 45 min using 90 μM
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29 PMEG (A) or 300 μM PMEDAP (C). Dependence of the transport efficiency on extracellular
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31 concentration of PMEG (B) and PMEDAP (D) was measured at t = 30 min.
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37 Fig. 4. Intracellular phosphorylation of [³H]PMEG and [³H]PMEDAP in sensitive (A,C) and
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39 resistant (B,D) CCRF-CEM cells after 24 h incubation with 2.5 μM PMEG and 15 μM
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41 PMEDAP (10 μCi / 10⁶ cells). Representative chromatograms out of two independent
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43 experiments performed with the same results are shown. Identity of all peaks was confirmed
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45 using the authentic standards.
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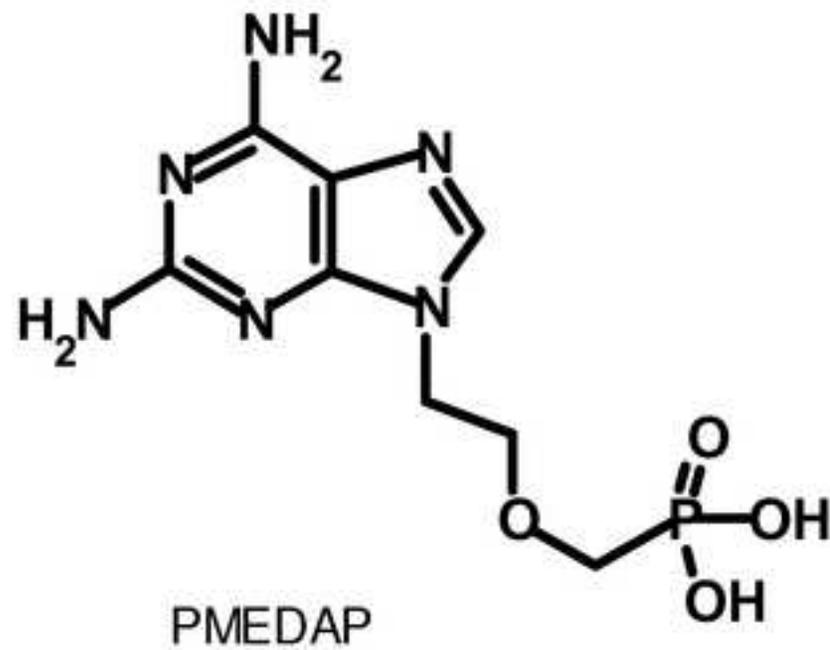
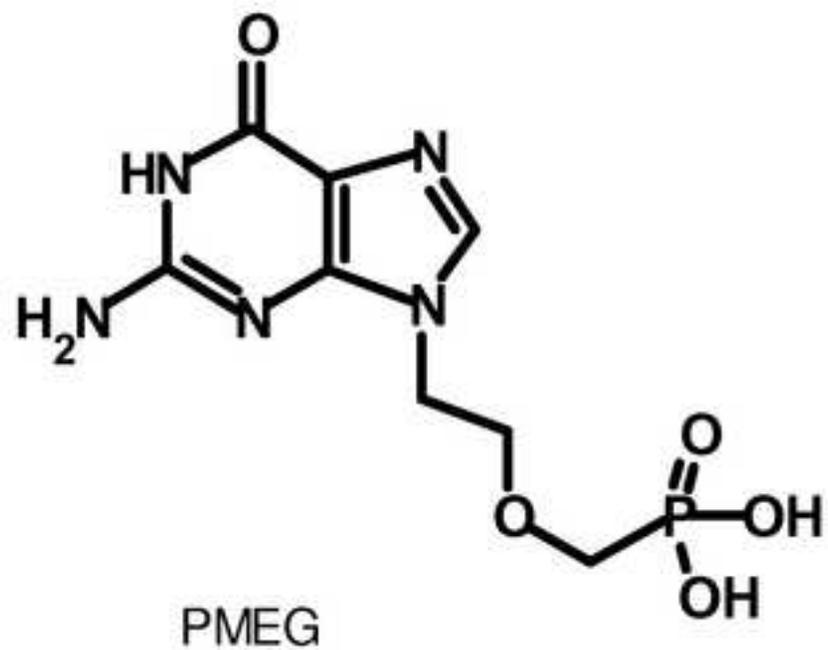
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52 Fig. 5. Expression of GUK1 and AK2 in sensitive and resistant CCRF-CEM cells. mRNA
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54 expression (A) was quantified by RT-qPCR and normalized to reference genes. Data represent
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56 means out of 3-4 independent experiments ± SD. * p < 0.05, ** p < 0.01 (One Way ANOVA
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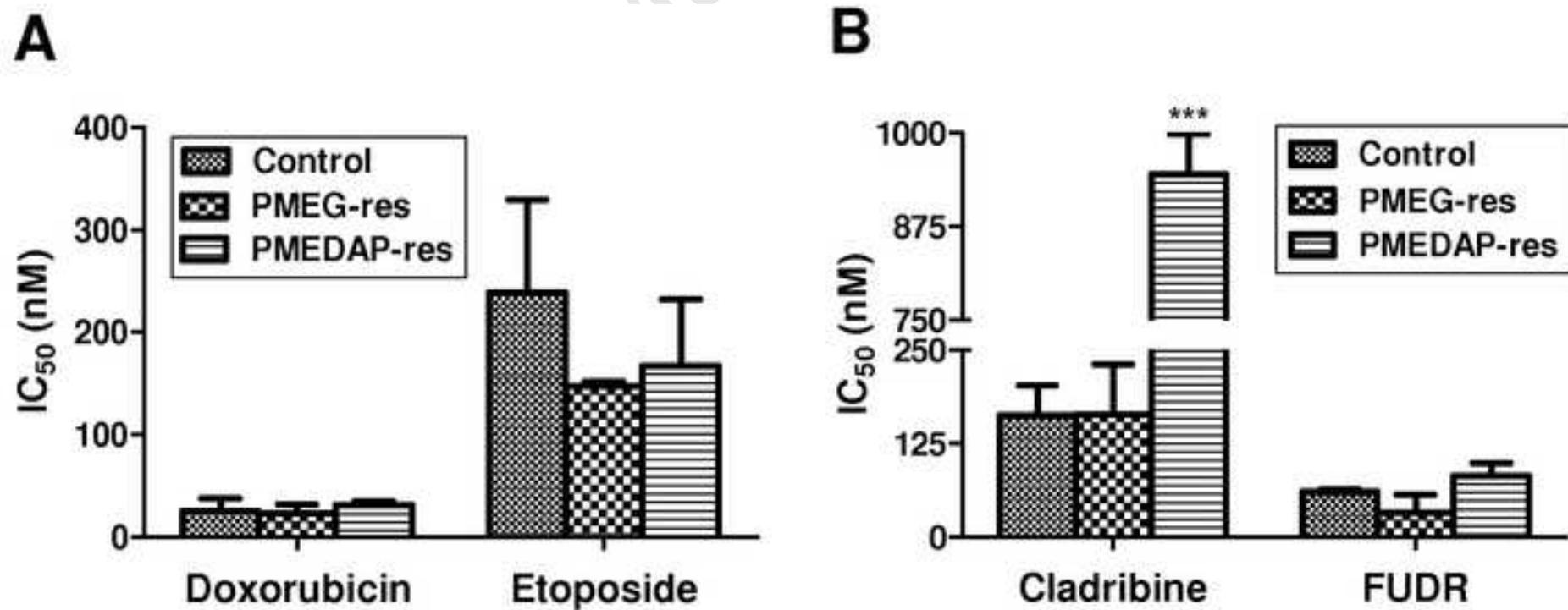
1 with Tukey's post hoc test). Protein levels of GUK1 and AK2 were detected by
2 immunoblotting (B). Blots are representative of two independent experiments performed.
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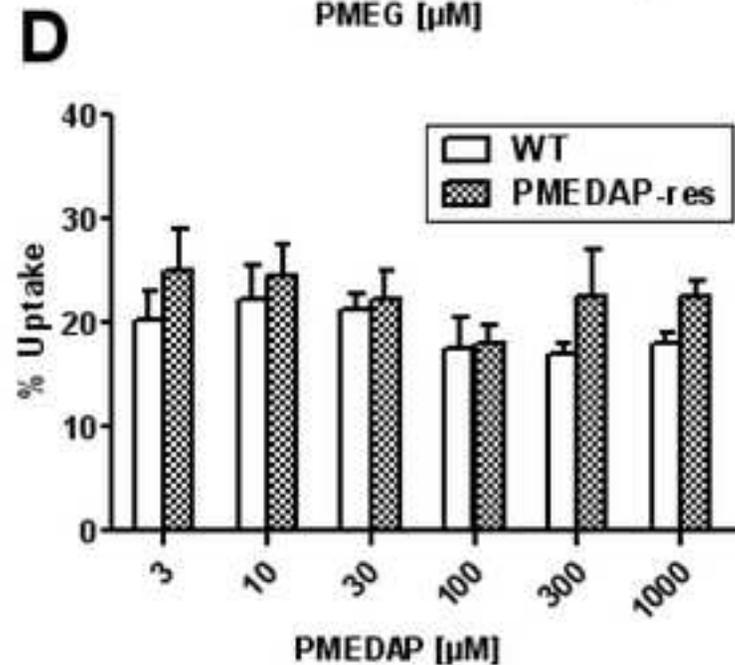
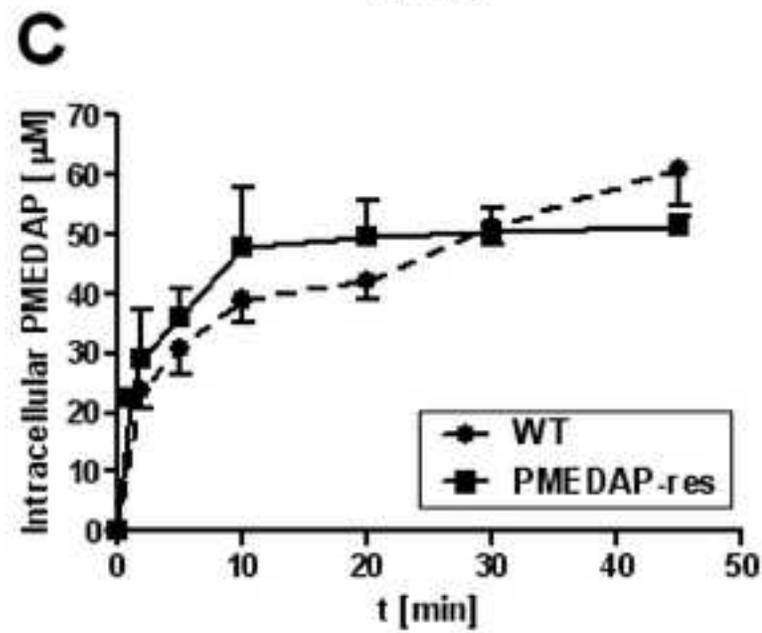
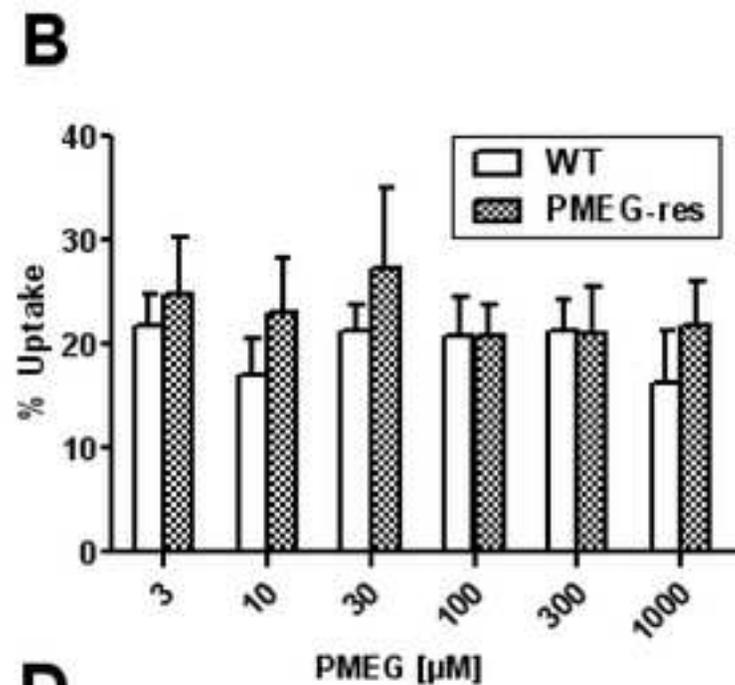
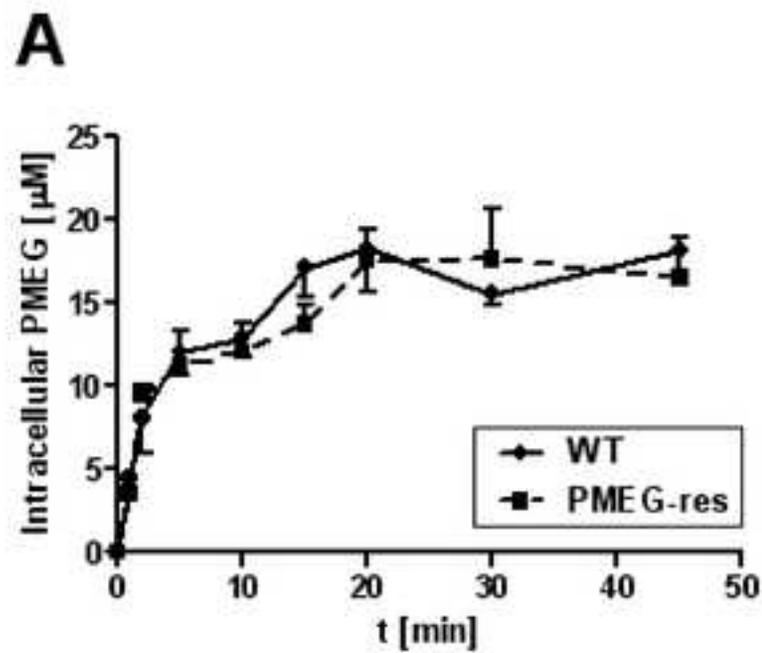
7 Fig. 6. Amino acids alignment of GUK 1 isolated from PMEG sensitive (GUK1 wt) and
8 PMEG-resistant (GUK PMEG-res) CCRF-CEM cells. Two point mutations, identified in
9 GUK from PMEG-resistant cells are shown in a grey box.
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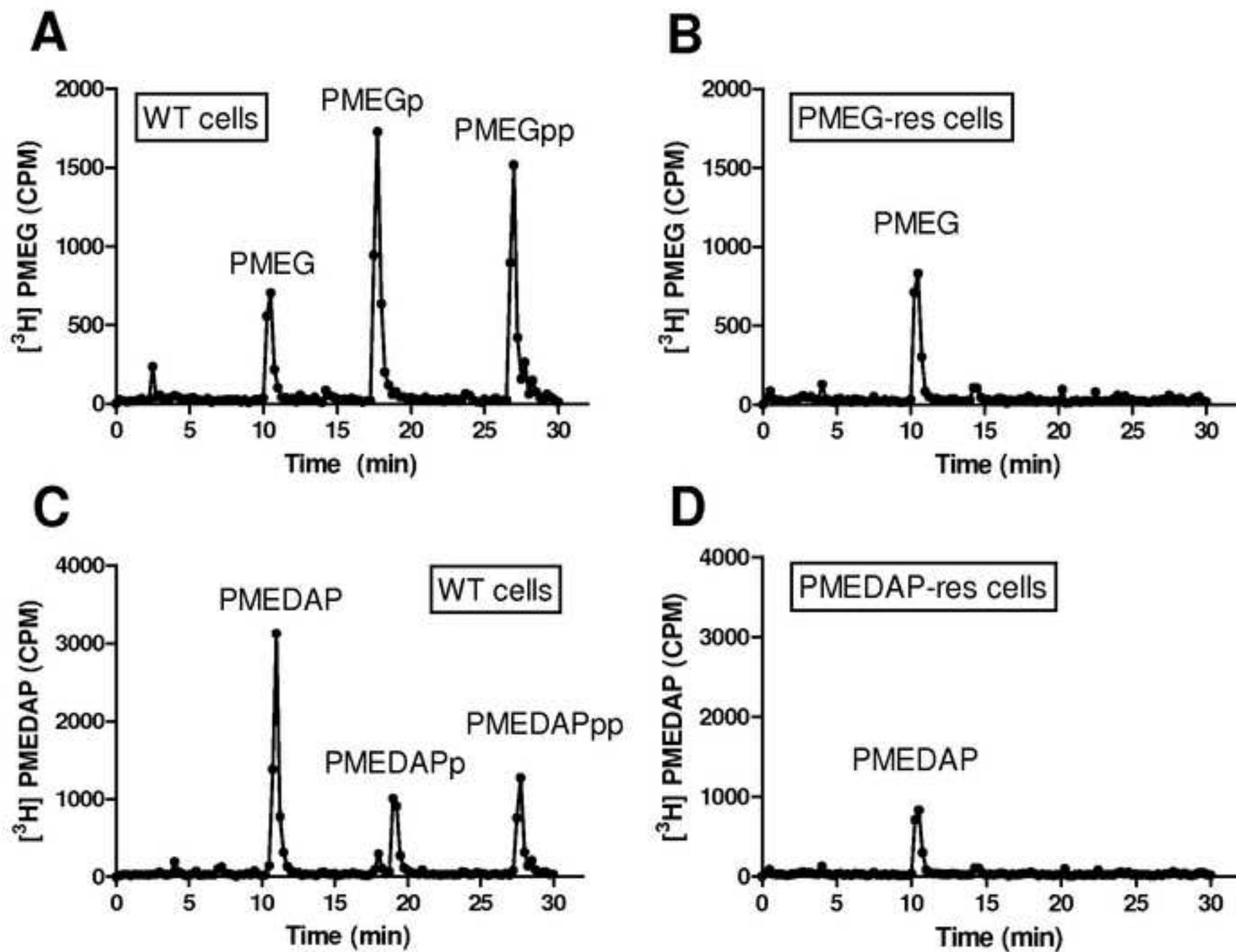
16 Fig. 7. Immunoblot analysis confirming the overexpression of GUK1 in PMEG-resistant cells
17 transfected with wtGUK (lanes 1,2), mutant SV/NF GUK (lanes 3,4) and mock transfected
18 cells (lane 5). Antibodies against both HA-tag (panel A) and GUK protein (panel B) yielded
19 the same results.
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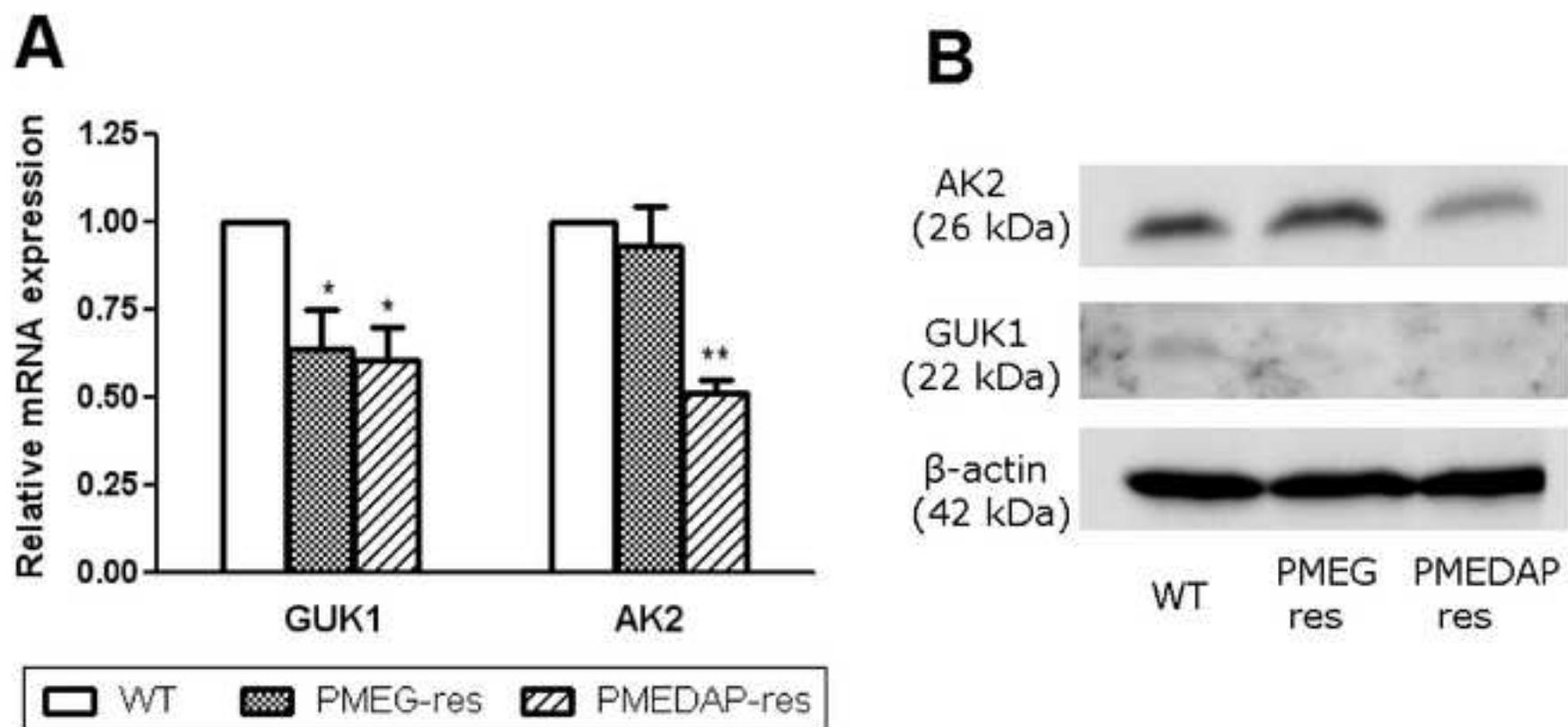
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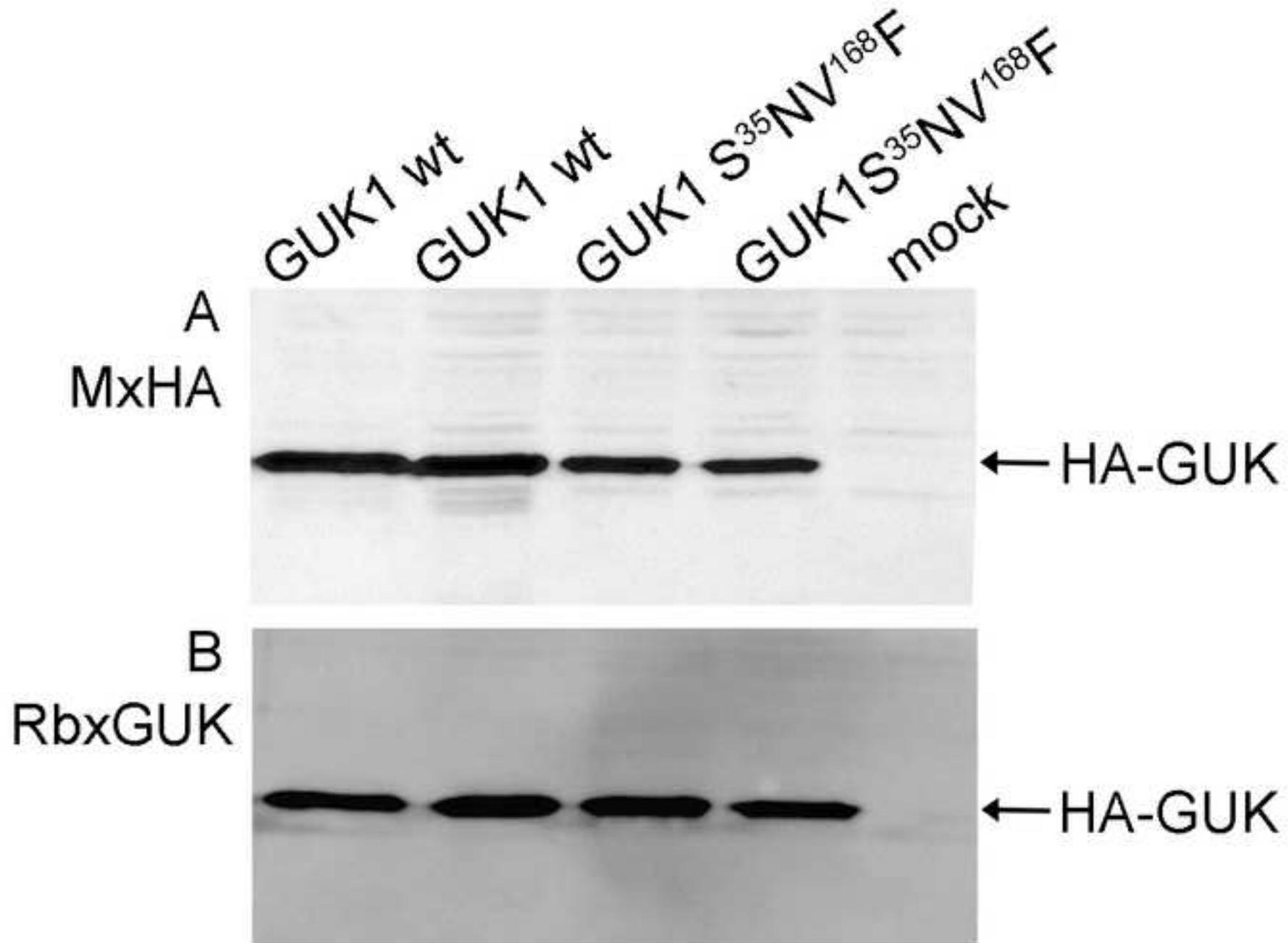






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GUK1 wt	¹ MSGPRPVVLSGPSGAGKSTLLKRLLEHSGIFGF S VSHTTRNPRPGEENGKDYYFVTREV
GUK1 PMEG-res	¹ MSGPRPVVLSGPSGAGKSTLLKRLLEHSGIFGF N VSHTTRNPRPGEENGKDYYFVTREV
GUK1 wt	⁶¹ MQRDIAAGDFIEHAEFSGNLYGTSKVAVQAVQAMNRCVLDVDLQGVRNIKATDLRPIYI
GUK1 PMEG-res	⁶¹ MQRDIAAGDFIEHAEFSGNLYGTSKVAVQAVQAMNRCVLDVDLQGVRNIKATDLRPIYI
GUK1 wt	¹²¹ SVQPPSLHVLEQRLRQRNTETEEESLVKRLAAAQADMESSEKPEGLFDV V IINDSLDQAYAE
GUK1 PMEG-res	¹²¹ SVQPPSLHVLEQRLRQRNTETEEESLVKRLAAAQADMESSEKPEGLFDV F IINDSLDQAYAE
GUK1 wt	¹⁸¹ LKEALSEEIKKAQRTGA
GUK1 PMEG-res	¹⁸¹ LKEALSEEIKKAQRTGA



Resistance to PMEG due to mutations in guanylate kinase (GUK). Transfection of resistant cells with wtGUK restores their phosphorylating activity and sensitivity to the cytotoxic effects of PMEG.

