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Point mutations in human guanylate kinase account for acquired resistance to anticancer nucleotide analogue PMEG

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ABSTRACT

Acyclic nucleotide analogue PMEG represents promising drug candidate against lymphomas. In the present work we describe the ability of PMEG to induce resistance and we elucidate the mechanisms involved in this process. CCRF-CEM T-lymphoblastic cells resistant to either PMEG or its 6-amino congener PMEDAP were prepared and assayed for the expression of membrane transporters, PMEG and PMEDAP uptake and intracellular metabolism. Genes for guanylate kinase (GUK) and adenylate kinase (AK) isolated from PMEG- and PMEDAP-resistant cells were sequenced and cloned into mammalian expression vectors. PMEG-resistant cells were transfected with GUK vectors and catalytic activities of GUKs isolated from PMEG-sensitive and resistant cells were compared. PMEG phosphorylation to PMEG mono- and diphosphate was completely impaired in resistant cells. GUK obtained from PMEG-resistant cells revealed two point mutations $S^{35}N$ $V^{168}F$ that significantly suppressed its catalytic activity. Transfection of resistant cells with wtGUK led to the recovery of phosphorylating activity as well as sensitivity towards PMEG cytotoxicity. No differences in PMEG uptake have been found between sensitive and resistant cells. In contrast to GUK no changes in primary sequence of AK isolated from PMEDAP resistant cells were identified. Therefore, resistance induced by PMEDAP appears to be conferred by other mechanisms. In conclusion, we have identified GUK as the sole molecular target for the development of acquired resistance to the cytotoxic nucleotide PMEG. Therefore, PMEG is unlikely to cause cross-resistance in combination therapeutic protocols with most other commonly used anticancer drugs.

1. Introduction

Nucleoside and nucleotide analogues rank among clinically important drugs in anticancer chemotherapy. Although acyclic nucleoside phosphonates (ANP) have now been predominantly recognized as efficient antiviral agents [1], their anticancer potency is also of interest [2]. 9-[2-(Phosphonomethoxyethyl)-guanine (PMEG) and 9-[2-(phosphonomethoxyethyl)diaminopurine (PMEDAP) (Fig. 1) represent the ANP with enhanced cytotoxic properties and possible use as novel antitumor compounds [3,4]. PMEG, in a form of a double prodrug GS-9219, has been previously shown to be active against Non-Hodgkin's lymphoma in dogs [5], while PMEDAP significantly prolonged survival of SD-rats with spontaneous lymphoma [6]. The advantage of ANP over conventional nucleotides lies in the chemical and metabolic stability of phosphonic bond. ANP bypass first-step phosphorylation, nevertheless they still require phosphorylation to ANPp and ANPpp to be active [7]. These reactions are catalyzed by specific nucleoside monophosphate kinases (NMPK) and relatively non-specific nucleoside diphosphate kinase (NDPK). We have previously demonstrated that PMEG is phosphorylated by guanosine monophosphate kinase (guanylate kinase, GUK) [8] while PMEDAP is activated by the mitochondrial isoform of adenosine monophosphate kinase (adenylate kinase 2, AK2) but not its cytosolic AK1 counterpart in L1210 cells [9]. These studies also indicated that both PMEG and PMEDAP were much weaker substrates for their respective phosphorylating enzymes compared to the natural substrates GMP and AMP, respectively.

Acquired resistance to chemotherapy upon prolonged or repeated administration is a serious issue complicating the treatment. Understanding the mechanisms leading to its develop-

Abbreviations: PMEG, 9-[2-(phosphonomethoxyethyl)guanine]; PMEDAP, 9-[2-(phosphonomethoxyethyl)diaminopurine]; PMEA, 9-[2-(phosphonomethoxyethyl)adenine]; GUK, guanylate kinase; AK, adenylate kinase; GMP, guanosine monophosphate; AMP, adenosine monophosphate; ANP, acyclic nucleoside phosphonate; CdA, 2-chloro-2′-deoxyadenosine (cladribine); FUDR, 5-fluoro-2′-deoxyuridine.

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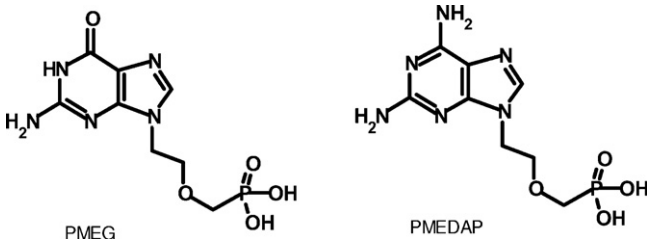

Fig. 1. Structures of 9-[2-(phosphonomethoxyethyl)guanine] (PMEG) and 9-[2-(phosphonomethoxyethyl)diaminopurine] (PMEDAP).

ment is crucial for designing strategies how to prevent or delay its onset as well as for predicting possible cross-resistance with other chemotherapeutics used within the same therapeutical protocol. Frequently, resistance occurs as a consequence of decreased intracellular concentration of the drug due to up-regulation of drug efflux proteins such ATP-binding cassette transporters (P-gp, MRP1, MRP4, MRP5) [10]. While nucleotides are not recognized as P-gp substrates, there is some evidence that MRP4 and/or MRP5 transporters may play role in resistance to 9-[2-(phosphonomethoxyethyl]adenine (PMEA) [11] and PMEDAP [12]. Another possible cause of the resistance that has been described in some anticancer nucleosides is represented by the defective metabolic activation, i.e. phosphorylation, by the individual kinases [13] or enhanced deactivation by nucleotidases [14]. Other means of chemoresistance development include alterations in various signaling pathways such as protein kinase signaling [15].

This work aims to elucidate the mechanisms responsible for the development of resistance in CCRF-CEM lymphoblastic cells following long-term exposure to PMEG. Emphasis has been placed on the role of intracellular transport and metabolism. The question whether there might be interferences with the cytostatic efficiency of other commonly used chemotherapeutics has also been addressed.

2. Methods

2.1. Materials

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

2.2. Cell culture

CCRF-CEM cells (ATCC CCL 119) were cultured under a humidified atmosphere containing 5% CO_2 at 37 °C. They were grown in T-25 flasks in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 200 μg/ml of streptomycin, 200 U/ml of penicillin G and 4 mM glutamine. Resistant cells were obtained by continuous exposure of the cells to increasing concentrations of PMEG or PMEDAP starting at their respective GIC_{50} (1 μM and 10 μM), and reaching 90 μM and 300 μM, respectively after 12 months. Cells were subcultured twice a week by centrifugation and fresh media with the compounds were added each time. Cell growth and viability was monitored using Countess® Automated Cell Counter (Invitrogen, Paisley, UK) following Trypan blue (0.4%) staining.

2.3. Cytotoxicity evaluation

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

2.4. Intracellular transport of [^{3}H]-PMEG and [^{3}H]-PMEDAP

CCRF-CEM cells were washed by centrifugation (250 × *g*, 5 min) in PBS and resuspended in RPMI medium w/o any additives. The cell suspension was distributed into microtubes in 450-μl aliquots and 50 μl [^{3}H]PMEG or [^{3}H]PMEDAP was added to the desired concentration. Incubation was done at 37 °C in a controlled CO_2-incubator using a rotary stirrer. At indicated time intervals the uptake process was terminated by centrifugation at 5300 × *g* for 1 min through an oil layer (a mixture of silicone and mineral oil at final specific density of 1.05 g/ml of 150 μl volume). The cell sediment was washed by centrifugation (5300 × *g*, 1 min) in 1 ml PBS, solubilized with Soluene® tissue solubilizer o/n and radioactivity was counted in a toluene scintillator (4 ml per sample). The intracellular volume of CCRF-CEM for calculation of the actual cytoplasmic concentration of PMEG and PMEDAP was 3.38 μl/10^{7} cells.

2.5. Intracellular metabolism of [^{3}H]PMEG and [^{3}H]PMEDAP in sensitive and resistant cells

The cells were washed with PBS, resuspended in 20 ml of RPMI growth medium at a concentration of 1 × 10^{6}/ml and incubated with 2.5 μM [^{3}H]PMEG (200 μCi) or 15 μM [^{3}H]PMEDAP (200 μCi) in a CO_2-incubator for 24 h at 37 °C. Cells were then washed in 1 ml PBS and pelleted by centrifuging at 250 × *g* for 1 min. The sediment was resuspended in 200 μl of deionized water and subjected to three freeze-thaw cycles. 200 μl of 10% trichloroacetic acid (TCA) was then added. After 10 min of vigorous shaking at 4 °C the precipitate was sedimented at 11,000 × *g* (5 min). TCA was extracted from the supernatant with 400 μl of a mixture of 1,1,2-trichloro-1,2,2-trifluoroethane–trioctylamine (4:1, v/v). The two phases were separated by centrifugation at 11,000 × *g* for 5 min and 50 μl of the upper aqueous layer was applied to Supelcosil LC-18 T HPLC column (150 mm × 4.6 mm, 3 μm) and analyzed for PMEG and PMEDAP metabolites. Elution buffer C contained 50 mM KH_2PO_4 and 3 mM tetrabutylammonium hydrogensulphate at pH 3.1. Buffer D was identical with C except for the addition of 30% (v/v) acetonitrile. The column was eluted with a linear gradient from 15% to 60% of buffer D for 25 min at the flow rate of 1 ml/min. 0.5-ml fractions were collected and radioactivity was counted in an aqueous scintillator (4 ml per sample). PMEG, PMEGp, PMEGpp, PMEDAP, PMEDAPp and PMEDAPpp were identified with the aid of authentic standards.