Original Article

Experimental Therapy with 9-[2-(Phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP): Origin of Resistance

(*MRP4* / *MRP5* / PMEDAP resistance)

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Abstract. The role of *MRP4* and *MRP5* transporters in the acyclic nucleoside phosphonate PMEDAP efflux was studied *in vitro* (CCRF-CEM cells) and *in vivo* (spontaneous transplantable T-cell lymphoma of SD/Cub inbred rats). The increased resistance against the cytostatic agent PMEDAP during long-term treatment was found to be associated with over-expression of *MRP4* and *MRP5* genes. The course of both gene activation differs significantly. While the *MRP5* function is important in the onset of PMEDAP resistance, the intensity of the relative *MRP4* gene expression increases rather continuously. Our data indicate cooperative acting of both *MRP4* and *MRP5* genes during the PMEDAP resistance development.

Introduction

Acyclic nucleoside phosphonates exhibit antiviral, cytostatic, antiparasitic and immunomodulatory effects (Holý, 2003). Among these nucleotide analogues 9-[2-(phosphonomethoxy)ethyl] derivative of 2,6-diaminopurine (PMEDAP) displays extensive antiviral and cytostatic activity (Veselý et al., 1990; Holý, 2003). This compound inhibits DNA synthesis and growth of L1210 mouse leukaemia cells *in vitro* and their DNA synthesis (Veselý et al., 1990) and is very effective in the treatment of lymphoblastic leukaemia/lymphoma of Sprague-Dawley rats (Otová, et al., 1997). PMEDAP is phosphorylated by cellular kinases to their diphosphate (analogue of nucleoside 5'-triphosphate) (Krejčová et al., 2000; Horská et al., 2006), which strongly inhibits replicative DNA polymerase δ (Kramata et al., 1996). This inhibition might be the basis for the cytostatic activity of this compound. The DNA replicative mechanism is able to eliminate the DNA damage caused by incorporation of the analogues at the end of the growing DNA chain by the action of pol δ and pol ε associated 3'- 5'exonuclease (Kramata et al., 1998; Birkuš et al., 1999). The effect of PMEDAP and other nucleoside phosphonates on the cell cycle and their capability to induce apoptosis was investigated on human leukaemia cell lines MOLT-4, HL-60 and ML-1 (Franěk et al., 1998). The cell growth experiments with HL-60 and MOLT-4 lines clearly showed that when using low concentrations of the analogue, the changes in the cell growth rate are fully reversible. Cell death occurs at higher drug concentrations only (Franěk et al., 1998).

Antitumour activity of compound PMEDAP has been studied in detail on a model of spontaneous T-cell lymphoma in inbred SD/Cub rats (Otová, et al., 1997; 1999; 2002). A significant therapeutic effect has been described after treatment with 16 daily doses of PMEDAP at 5 mg/kg applied to the vicinity of growing lymphoma (Bobková et al., 2000). A decrease of the lymphoma weight during PMEDAP administration was accompanied by suppression of mitotic activity of neoplastic cells and increased chromatin condensation as well as

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Abbreviations: DTX – docetaxel, MRP (1–5) – multiple drug resistance protein, PMEA – 9-[2-(phosphonomethoxy)ethyl]adenine, PMEDAP – 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine, RQ-RT-PCR – real-time quantitative reverse transcriptase-polymerase chain reaction, SD/Cub – Sprague-Dawley inbred rats/Charles University Biology.

by increased nuclear DNA fragmentation resulting in induction of apoptosis (Otová et al., 1999) in *in vivo* growing lymphomas. Another study has shown that the therapeutic effect of PMEDAP depends on the phenotype of the individual neoplasia (Bobková et al., 2000).

Antitumour efficacy of the combined therapy with PMEDAP and docetaxel (DTX) was studied in an *in vivo* model of s.c. transplanted Sprague-Dawley (SD/Cub) rat T-cell lymphoma (Bobková et al., 2001). The effect of combined treatment of DTX with PMEDAP was significantly higher than that of DTX or PMEDAP alone (Bobková et al., 2001). The s.c. administration of DTX into the vicinity of the growing lymphoma combined with i.p. administration of PMEDAP was the most efficient therapy. Preliminary results from our laboratory show that the effect of long-term *in vivo* treatment with PMEDAP is limited. Its high antiproliferative activity at the beginning of the treatment is followed by a decrease of the therapeutic efficacy of the compound. Moreover, other studies show that *in vitro* resistance against structurally closely related adenine congener PMEA {9-[2-(phosphonomethoxy)ethyl]adenine} is due to over-expression and amplification of the *MRP4* gene and correlates with ATP-dependent efflux of the drug from cells (Schuetz et al., 1999; Sampath et al., 2002). PMEA does not appear to be a substrate for the P-gp (Hatse et al., 1998), nor does it interact with typical substrates for *MRP1–3* (Schuetz et al., 1999**).** PMEA resistance provided by *MRP4* was also confirmed in subsequent studies by Lee et al. (2000).

In this study we demonstrate that the resistance to PMEDAP is closely connected with the expression of both *MRP4* and *MRP5* genes.

Material and Methods

Drug

9-[2-(Phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP), an acyclic nucleoside phosphonate, was prepared according to Holý et al. (1989).

Cells

Human T-cell acute lymphoblastic leukaemia cells (CCRF-CEM, ATCC CCL 119), which were chosen as a model of haematological malignancy, were cultivated in RPMI 1640 medium (Sigma, Saint Louis, MO*)* enriched with 10% calf foetal serum (PAA, Pasching, Austria). Selection of PMEDAP-enhanced resistance in CCRF-CEM cells was achieved by 2-year exposure of cell cultures with 3.5 μM and/or 7 μM PMEDAP (IC_{50}). The cell culture proliferation activity was monitored by XTT-test (Roche, Diagnostics GmbH, Mannheim, Germany).

Experimental animals

Two to three month old males of the Prague subline of Sprague-Dawley inbred rats (SD/Cub) were used for our experiments. The rats were bred under conventional conditions with commercial granule food (Bergman TM2-CH, Woodland Hills, CA) and tap water *ad libitum*. Five animals in each treated and control group of the same age were used. Animal handling and experimental protocol were undertaken as per guidelines of the Institutional Ethical Committee for Animal Use.

Neoplasia

Spontaneous transplantable T-cell lymphoma is a highly genetically defined model of spontaneous haematological malignancy (Otová et al., 2002). In this study lymphoma SD 10/96 (68^{th} passage) was used. In all experiments, suspension of 10^6 lymphoma cells in PBS (saline) was injected subcutaneously into the right flank of anaesthetized rat.

Drug administration

Growing CCRF-CEM cells were cultivated for a period of 5 weeks in the presence of 1 μM PMEDAP. Samples for RQ-RT-PCR were recovered every 7 days and at the same time the medium was changed for the fresh one. Medium was also changed every week 72 h before recovering the cells for examination. Control, non-treated cells were maintained to proliferate in the same way. The cell proliferation was evaluated by XTT-test.

In *in vivo* experiments PMEDAP was injected subcutaneously in the vicinity of the growing tumour in each animal in the treated group at a dose of 5 $mg.kg^{-1}$, once daily (1 x d) for 5 consecutive days, followed by 2-day cessation. Administration of the drug started 3 days after inoculation of tumour cells (1^{st} passage) and continued during the next 21 days. Twenty-four h after the last injection of PMEDAP autopsy was made and lymphoma weight was measured. The same administration schedule was repeated three times (2^{nd} passage – 4^{th} passage). The amount of 10^7 of cells was taken from each tumor (separately from the treated and the control group). The quantity of 10^6 of cells was used for the new passage and the rest of cells were examined by RQ-RT-PCR.

Quantification of gene expression (RQ-RT-PCR)

Total RNA was extracted from human and rat cells by the modified method described by Gauthier et al. (1997). cDNA was synthesized using MoMLV reverse transcriptase (Gibco BRL, Carlsbad, TX) according to manufacturer's instructions (random priming, out of 5 μg of RNA template).

Real-time PCR was performed in the LightCycler™ rapid thermal cycler system (Roche). SYBR Green (FMC BioProducts, Rockland MA) (Morrison et al., 1998) was used for quantification of *MRP5* and β-actin genes (used for *in vivo* experiment analysis). Another housekeeping gene β2-microglobulin (used for *in vitro* experiment analysis) hybridization probes were used for quantification. For the *MRP4* gene, hydrolysis probes

Table 1. Sequences of primers and probes

MRP4 hum - sense	5′ TGGATTCTGTGGCTTTGAACAC 3′
MRP4 hum - antisense	5′ AGCCAAAATGAGCGTGCAA 3′
MRP4 hum - probe	5′ 6FAM-CGTACGCCTATGCCACGGTGCTG 3′
MRP4 rat - sense	5′ TTCAGGACAGCCATACCTAATAAAAA 3′
MRP4 rat - antisense	5′ GCCTTTGCGTCATGTTCAGTAG 3′
MRP4 rat - probe	5′ 6FAM-TGTGCTGTGTTAGCGCCAGCCAA 3′
B2MF - sense	5′ CCAGCAGAGAATGGAAAGTC 3′
B2MA - antisense	5′ GATGCTGCTTACATGTCTCG 3′
B2M3FL - probe	5′ TTCTTCAGTAAGTCAACTTCAATGTCGGA -- FL 3′
B2M5LC - probe	5′ LC Red640-ATGAAACCCAGACACATAGCAATTCAG--PH 3′
MRP5 sense	5′ GCTGACCATTGCCCATCG 3′
MRP5 antisense	5′ GTCGTTGGACAGAAGGACCG 3′
β actin - sense	5′ TTACTGCCCTGGCTCCTAGCA 3′
β actin - antisense	5′ GGGCCGGACTCATCGTACTC 3′

were designed. Specific primer pairs were designed as shown in Table 1.

The PCR mixture and cycling conditions were used as follows:

MRP4 gene (human cell line): the reaction mixture (20 µl) contained 0.3 µg of cDNA, 1 U of Platinum Taq DNA Polymerase (Gibco BRL), each primer 0.5 µmol.l^{-1}, 0.1 µmol.l^{-1} of probe, 4 mM $MgCl_2$, 0.2 mM dNTPs, 5 µg of BSA (bovine serum albumin, Sigma); the Light-Cycler programme consisted of 94°C for 2 min followed by 45 cycles at 95°C for 20 s, 62°C for 1 min.

MRP4 gene (rat): the reaction mixture (20 µl) contained 0.3 µg of cDNA, 1 U of Platinum Taq DNA Polymerase, 0.5 µmol.l^{-1} of each primer, 0.1 µmol.l^{-1} of probe, 4 mM $MgCl_2$, 0.2 mM dNTPs, 5 µg of BSA; 94°C for 2 min followed by 45 cycles at 95°C for 20 s, 60°C for 1 min.

MRP5 gene (human and rat): the reaction mixture (20 µl) contained 0.2 µg of cDNA, 1 U of Platinum, 0.2 µl of SYBR Green (2 x 10^{-4} of stock concentration diluted by DMSO), Taq DNA Polymerase, 0.25 µmol.l^{-1} of each primer, 2 mM $MgCl_2$, 0.2 mM of dNTPs, 5 µg of BSA; 94°C for 3 min; followed by 40 cycles at 95°C for 5 s, 62°C for 40 s and 72°C for 12 s.

β2-Microglobulin gene: the reaction mixture (20 µl) contained 0.2 µg of cDNA, 0.2 µl of Platinum Taq DNA Polymerase, 0.5 µmol.l^{-1} of each primer, 0.2 µmol.l^{-1} of probe, 3 mM $MgCl_2$, 0.2 mM dNTPs, 5 µg BSA; 95°C for 2 min and 30 s followed by 45 cycles at 95°C for 3 s, 62°C for 10 s and 72°C 5 s.

β-Actin gene: the reaction mixture (20 µl) contained 0.3 µg of cDNA, 0.2 µl of Platinum Taq DNA Polymerase, 0.5 µmol.l^{-1} of each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µl of SYBR Green (2 x 10^{-4} of stock concentration diluted by DMSO), 5 µg of BSA; 95°C for 3 min followed by 50 cycles at 95°C for 5 s, 68°C for 30 s and 72°C for 15 s.

Levels of the gene transcripts were quantified by the ratio of the intensity of the target signal over the intensity of the β2-microglobulin or β-actin internal standard in the same pentaplex PCR reaction.

Statistics

The Student's *t*-test was used for the statistical comparison and P values ≤ 0.05 were considered statistically significant.

Results and Discussion

In vitro experiment

The relative expression of *MRP4* and *MRP5* genes in CCRF-CEM cell cultures after long-term exposure with PMEDAP (3.5 µmol.l^{-1} and/or 7 µmol.l^{-1}) was compared with the expression of both genes in control cells. The data show that the constitutive expression of the *MRP5* gene is significantly higher in the control cells compared to the cells with PMEDAP-induced resistance ($P < 0.01$; Fig. 1a). On the other hand, in comparison with the control cells, the *MRP4* gene expression increases significantly in 7 µM PMEDAP-treated cells, but not in 3.5 µM PMEDAP-treated cells ($P < 0.01$; Fig. 1a, 1b).

In the next experiment we followed the relative expression of *MRP4* and *MRP5* genes in this cell line treated with 1 µM PMEDAP during five passages lasting 35 days. The course of the *MRP4* gene expression shows ($P < 0.01$; Fig. 2a) the highest intensity during the first passage, exceeding the expression in the control cells more than ten times, and was accompanied by decreased proliferation (data not shown). Surprisingly, the *MRP4* gene expression was going down during the next three passages (14–28 days), while the proliferation was going up, reaching the intensity comparable with the *MRP4* expression in the control cells followed by significant

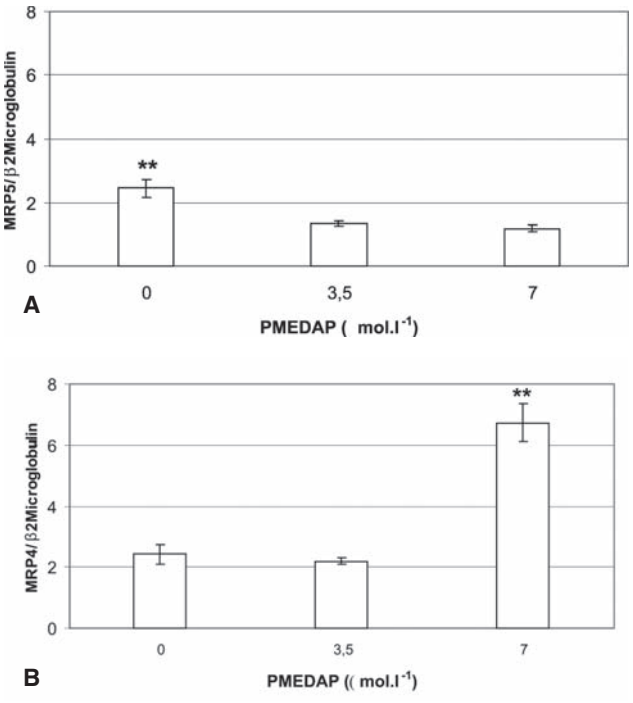

Fig. 1. Relative expression of *MRP5* and *MRP4* genes in CCRF-CEM cells after long-term treatment with PMEDAP. Expression of *MRP5* (*a*) and *MRP4* (*b*) after 2-year exposure to PMEDAP (3.5 and 7 μmol.l^{-1}) is related to expression of β-microglobulin.
Student's *t*-test: ** = P < 0.01

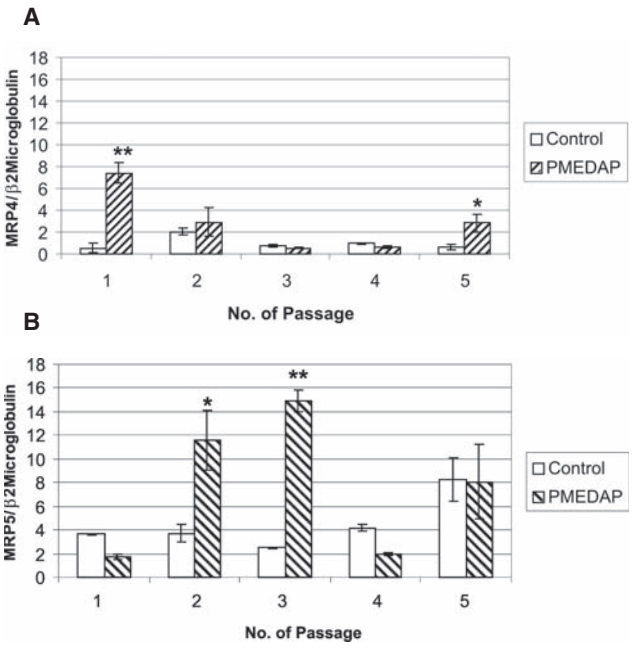

Fig. 2. Relative expression of *MRP5* and *MRP4* genes in CCRF-CEM cells after long-term treatment with PMEDAP. Expression of *MRP4* (*a*) and *MRP5* (*b*) after 35-day (5 passages) exposure to PMEDAP (1 μmol.l^{-1}) is related to expression of β-microglobulin. Control – white bar, PMEDAP-treated – hatched bar.
Student's *t*-test: ** = P < 0.01; * = P < 0.05

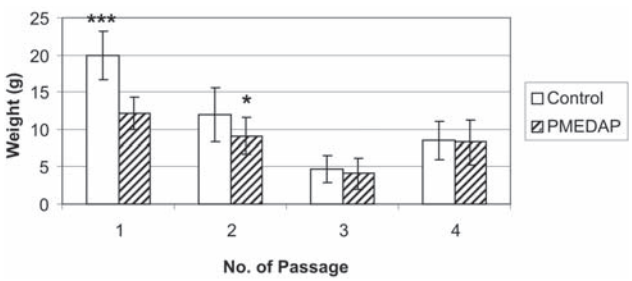

Fig. 3. Weight of lymphomas of PMEDAP-treated SD/Cub rats. PMEDAP was injected subcutaneously in the vicinity of the growing tumour in each animal in the treated group at a dose of 5 mg.kg^{-1}, once daily (1 x d) for 5 consecutive days, followed by 2-day cessation. Administration of the drug started 3 days after inoculation of tumour cells (1st passage) and continued during the next 21 days. Control – white bar, PMEDAP treated – hatched bar.
Student's *t*-test: *** = P < 0.001; * = P < 0.05

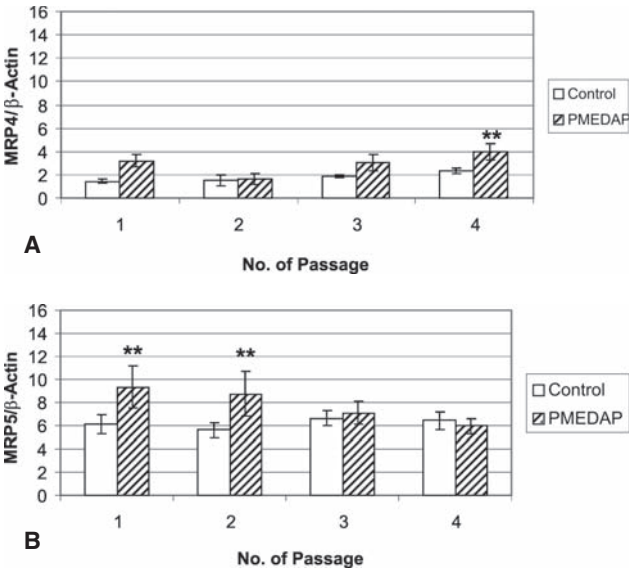

Fig. 4. Relative expression of *MRP5* and *MRP4* genes in PMEDAP-treated SD/Cub rats. Expression of *MRP4* (*a*) and *MRP5* (*b*) after PMEDAP treatment (as described in legend to Fig. 3) is related to expression of β-actin. Control – white bar, PMEDAP-treated – hatched bar.
Student's *t*-test: ** = P <0 .01

increase in the fifth passage (35 days; P < 0.05). The *MRP5* gene showed an entirely reverse course of the relative expression, reaching the peak value in the third passage (21 days, P < 0.01; Fig. 2b).

In vivo experiment

Relative values of *MRP4* and *MRP5* gene expression were monitored in SD-lymphoma cells in relation to the tumour weight of the experimental animals treated with PMEDAP (5 mg.kg^{-1}) during four passages (4 x 21 days). The data show (Fig. 3) that the lymphoma weight of the PMEDAP-cured rats was significantly lower dur-

ing the first ($P < 0.001$) and second passage ($P < 0.05$), and the susceptibility toward the drug was lost during the third and fourth passage. The intensity of the relative *MRP4* gene expression, estimated in parallel, increased continuously up to the fourth passage ($P < 0.01$; Fig. 4a). On the other hand, *MRP5* gene expression was increasing after the first passage and then, after the second passage, was going down and the levels during the third and fourth passage were the same as in the untreated rats ($P < 0.01$; Fig. 4b).

The role of *MRP4* and *MRP5* transporters in the acyclic nucleoside phosphonate efflux, mostly on highly resistant cell line CEM/PMEA-2, was intensively studied in the past (Schuetz et al., 1999; Wijnholds et al., 2000) and the results of these experiments, including other nucleotide-based therapeutics, were recently summarized (Sampath et al., 2002). In our study, we compared expression of both genes *in vitro* and *in vivo* in quite different experimental settings. We evaluated simultaneous relative *MRP4* and *MRP5* gene expression using low concentrations of acyclic nucleoside phosphonate PMEDAP in cell line CCRF-CEM which correspond to intracellular and tissue levels of the drug during the *in vivo* treatment of experimental animals (Otová, et al., 1997, 1999). Our data indicate a cooperative action of both genes during the long-term drug administration *in vitro* and/or *in vivo*. This action is characterized *in vitro* by relatively high expression of *MRP4* immediately after drug administration, while the *MRP5* is intensively expressed later in the third passage and then is suppressed up to the constitutive level. The *MRP5* suppression is accompanied by increasing *MRP4* expression. On the other hand, we did not find any changes in the *MRP5* and *MRP4* level in the cell lines with enhanced resistance towards 3.5 μM PMEDAP (0.5 x IC_{50}); the constitutive level of *MRP4* expression was higher when the cell line was resistant to 7 μM PMEDAP (IC_{50}) compared to wild-type CCRF-CEM cell line.

In *in vivo* experiments we followed relative *MRP4* and *MRP5* gene expression directly in the lymphoma cells treated with PMEDAP long-term at a therapeutic dose. The course of expression of both genes was similar to that in the CCRF-CEM cell line. In the first passage (21 days) *MRP4* and *MRP5* displayed considerably enhanced activity and then the intensity of *MRP5* expression was going down while that of *MRP4* rose.

In summary, in accordance with the previous findings our study shows that *MRP4* and *MRP5* participate in protection of cells and/or tissues against the cytotoxic effects of antineoplastic compound PMEDAP. The expression of both transporters is variable and cooperative, probably due to the fact that following long-term treatment, the drug exerts enhanced *MRP4* expression only, while the *MRP5* function is important in the onset of PMEDAP resistance.

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