Multidrug Resistance Protein 4 Protects Bone Marrow, Thymus, Spleen, and Intestine from Nucleotide Analogue–Induced Damage

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Abstract
Nucleoside-based analogues are mainstays in the treatment of cancer, viral infections, and inflammatory diseases. Recent studies showing that the ATP-binding cassette transporter, multidrug resistance protein 4, is able to efflux nucleoside and nucleotide analogues from transfected cells suggests that the pump may affect the efficacy of this class of agents. However, the in vivo pharmacologic functions of the pump are largely unexplored. Here, using Mrp4−/− mice as a model system, and the nucleotide analogue, 9′-(2′-phosphonylmethoxyethyl)-adenoine (PMEA) as a probe, we investigate the ability of Mrp4 to function in vivo as an endogenous resistance factor. In the absence of alterations in plasma PMEA levels, Mrp4-null mice treated with PMEA exhibit increased lethality associated with marked toxicity in several tissues. Affected tissues include the bone marrow, spleen, thymus, and gastrointestinal tract. In addition, PMEA penetration into the brain is increased in Mrp4−/− mice. These findings indicate that Mrp4 is an endogenous resistance factor, and that the pump may be a component of the blood-brain barrier for nucleoside-based analogues. This is the first demonstration that an ATP-binding cassette transporter can affect in vivo tissue sensitivity towards this class of agents. [Cancer Res 2007;67(1):262–8]

Introduction
Nucleoside-based drugs are mainstays in the treatment of cancer, viral infections, and inflammatory diseases. Factors that govern the intracellular accumulation of these agents may limit their efficacy by restricting penetration into tumors, inflammatory cells, and infected tissues, and by functioning as a pharmacologic barrier at anatomic sanctuary sites (1). This notion is well established for natural product anticancer agents, for which MRPl is an endogenous resistance factor, and P-glycoprotein restricts oral bioavailability and penetration into the brain and fetus (2–6). In contrast with natural product agents, the distribution and penetration into anatomic sanctuary sites, such as the apical (luminal) surfaces of kidney proximal tubules and brain capillaries, and the basolateral surface of hepatocytes (15–17). These properties suggest that MRPl may affect the accumulation of nucleoside-based agents into tissues, and possibly their penetration into sanctuary sites, and elimination from the body. However, with the exception of a report showing that Mrp4 restricts the penetration of topotecan into the brain and cerebrospinal fluid (16), little is known about the in vivo pharmacologic functions of the pump. Notably, the ability of MRPl to affect tissue chemosensitivity has not been explored to any extent.

Here, we investigate the in vivo pharmacologic function of MRPl, using Mrp4−/− mice as a model system, and PMEA, one of the best characterized drug substrates of MRPl, as a probe. The results show that Mrp4 protects the bone marrow, spleen, thymus, and intestine from PMEA-induced damage, and that the pump also restricts the penetration of PMEA into the brain. This is the first demonstration that an efflux pump is able to function as an in vivo resistance factor for a nucleoside-based agent.

Materials and Methods

Generation of Mrp4 gene–disrupted mice. A 500 bp fragment of the 5′ end of the mouse Mrp4 coding sequence was used to screen a mouse strain 129-derived λ phage genomic library. Five overlapping clones spanning 29 kb and including three upstream exons of the Mrp4 gene were isolated and characterized. A targeting vector was designed to delete sequences corresponding to amino acids 77 to 101 of Mrp4. To create the 5′ arm of the targeting vector, an EcoRI-Xhol fragment was cloned into corresponding sites of the Bluescript SK− vector (Stratagene, La Jolla, CA), the Xhol site was modified by the addition of an XbaI-SpeI-Xhol adaptor, and the fragment was reintroduced into the Smal and SpeI sites of Bluescript using an internal SpeI site and the SpeI site from the adaptor. The 3.6 kb fragment was then excised from Bluescript using flanking EcoRI and XbaI sites and inserted into corresponding sites of the PNT vector. To create the 3′ arm, a 3.7 kb XhoI-EcoRI fragment was cloned into the corresponding sites of the Bluescript vector, the XhoI site and a flanking NotI site were used to subclone the fragment into corresponding sites of PNT. The targeting vector was digested with NotI and linearized DNA was

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electroproated into strain 129-derived R1 embryonic stem cells. Individual colonies isolated following positive/negative selection with G418 and ganciclovir were screened by Southern blot analysis using a 5′ probe and genomic DNA digested with BamHI, and a 3′ probe and DNA digested with HindIII. The absence of randomly integrated vector sequences was confirmed by Southern blot analysis using a neo probe. Two correctly targeted ES cell lines were injected into C57BL/6 Blastocysts, and the blastocysts were implanted in pseudopregnant females. Male chimeric progeny were crossed with female C57BL/6J mice (bred in-house). Germ line transmission of the targeted allele was confirmed by Southern blot analysis and subsequent genotyping was accomplished by PCR analysis of tail DNA. The latter reaction was carried out in a single tube using three primers: 5′-ggggatcagtcctactgttcctga-3′, 5′-gggctagctgttctcggcagtcctactgttcctga-3′, and 5′-gggagctgcatcctgctctcggcagtcctactgttcctga-3′. The former two primers generated a 360 bp wild-type product, and the latter two generate a 270-bp product from the targeted allele.

Animal handling, blood chemistries, and hematology. Animals were maintained in the Fox Chase laboratory animal facility and housed in a temperature- and humidity-controlled environment under 12-h light/dark cycles. Mice were fed a standard rodent diet (Lab Diet 5013, PMI Nutrition, Brentwood, MO) and had free access to water. The Fox Chase Institutional Animal Care and Use Committee approved the protocol. Peripheral blood was obtained by orbital bleeding of anesthetized mice. Blood chemistry and hematologic variables were determined at Antech Diagnostics (Farmingdale, NY).

Immunoblot analysis. Crude membrane preparations were resolved by SDS-PAGE and proteins were transferred to nitrocellulose membranes. Mrp4 was detected using a previously described rabbit polyclonal Mrp4 antibody (18).

Histopathology and drug sensitivity analysis. Mrp4−/− mice on a mixed C57BL/6J × 129 background were used for histopathologic studies and drug sensitivity experiments. Histopathologic analysis of Mrp4-null mice revealed normal heart, lungs, thymus, spleen, bone marrow, forestomach, stomach, small intestine, pancreas, kidneys, ureter, bladder, testes, ovaries, uterus, and brain. For drug sensitivity experiments, age-matched male mice (n = 6) were treated with single i.p. injections of PMEA, and observed daily for a period of 2 weeks. For analysis of weight progression and WBC counts, groups of male mice (n = 4) were treated with PMEA at 2 g/kg body weight. Mice were weighed and blood samples (20 μL) were taken by orbital bleeding, for a period of 5 days. WBC counts were analyzed using a Coulter Z1 Series Particle Counter (Beckman Coulter, Miami, FL). For histologic analysis, mice were euthanized 3 days after being treated with 2 g/kg of PMEA. Tissues were harvested and fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with H&E.

Pharmacokinetic measurements. Adult male (~25 g body weight) C57BL/6J and Mrp4-null mice were used for all pharmacokinetic studies. Mice were anesthetized with a 3:1 mixture of ketamine hydrochloride/acepromazine maleate/xylazine hydrochloride (100:10:20 mg/mL), followed by the implantation of a right common carotid artery and a jugular vein cannula (0.28 mm inside diameter and 0.64 outside diameter; SCI Micro Medical Tubing), which were used for blood sampling and drug administration, respectively. The cannulas were secured and exteriorized at the back of the neck. Animals were conscious for the pharmacokinetic studies, having been allowed to recover for at least 12 h before being placed at the start of the study in individual metabolism cages, which facilitated collection of urine.

For single-dose pharmacokinetic experiments, groups (n = 6–8) of each mouse strain received either 5 or 25 mg/kg of PMEA, dissolved in isotonic saline in phosphate buffer (13.5 mmol/L KH2PO4 and 62.5 mmol/L Na2HPO4), by i.v. injection over 1 min. Serial blood samples (20 μL each) were collected at 2, 5, 15, 30, 60, 90, 120, 180, 240, 300, and 360 min, after which plasma was separated from blood and then stored at −80°C until analyzed by high-pressure liquid chromatography (HPLC) as described below. Urine was collected on dry ice for ~6 h, which enabled renal clearance to be estimated. The total volume of urine was measured and stored at −80°C until analyzed by HPLC. To examine the effects of probenecid on the pharmacokinetic properties of PMEA, a combined i.v. loading dose (20 mg/kg) and constant rate infusion (20 mg/kg/h) regimen of probenecid was administered to individual mice of both strains. This regimen was estimated to attain steady-state probenecid plasma concentrations of ~0.5 μg/mL. After 2 h of the constant rate infusion of probenecid, a single 25 mg/kg i.v. bolus dose of PMEA was administered. The probenecid infusion was continued for an additional 6 h during a blood sampling period analogous to that used for the single-dose PMEA study described above. Probenecid plasma concentrations were measured just prior to the administration of PMEA and at 360 min, the last time point.

For steady-state pharmacokinetic measurements of brain PMEA concentrations, specific steady-state dosing regimens of PMEA were designed to achieve plasma concentrations of 5 μg/mL for each mouse strain, based on the results from the single-dose PMEA pharmacokinetic studies. C57BL/6J wild-type mice received a loading dose of 3.83 mg/kg of PMEA and a constant rate infusion of 0.235 mg/kg/min for 3 h, whereas Mrp4−/− mice were given a loading dose of 3.82 mg/kg of PMEA and constant rate infusion of 0.217 mg/kg/min for 3 h. Approximately 30 μL of blood was collected at 30, 60, 120, and 180 min during the infusions, after which the animals were immediately sacrificed and brain tissue was harvested.
collected. Tissues were rapidly frozen with dry ice and stored at −80°C until analyzed by HPLC.

**HPLC analysis of PMEA and probenecid.** HPLC analysis of PMEA was based on a previously published method developed for human plasma that used a derivatization step and fluorescence detection (19). Plasma, urine, and brain tissue samples were prepared as follows for analysis of PMEA in plasma, each 10 μL aliquot was mixed with 100 μL of methanol by vortexing for 1 min. After centrifugation for 3 min at 15,000 rpm, the supernatant was evaporated under nitrogen at room temperature for 40 min. One hundred microliters of 0.5% chloroacetaldehyde (derivatization reagent) in 0.1 mol/L sodium acetate (pH 4.5) was added to the residue, and the solution was incubated at 90°C for 40 min followed by centrifugation at 15,000 rpm for 1 min. A 20 μL aliquot of the supernatant was injected onto the HPLC system. For analysis of PMEA in urine, each 10 μL aliquot was mixed with 990 μL of water, and 10 μL of the mixture was added to 100 μL of 0.5% derivatization reagent. The solution was then incubated and processed as described for plasma. For the analysis of PMEA in brain tissue, each aliquot of 60 μL of brain homogenate (10%, 1 g tissue:9 mL water) was centrifuged at 15,000 rpm for 5 min, followed by the addition of 100 μL of 0.5% derivatization reagent to 40 μL of supernatant. The solution was then incubated and processed as described for plasma.

PMEA was separated on a C8 column (5 μm, 150 × 4.6 mm; Phenomenex Hypersil 5) using a mobile phase of 15:85 acetonitrile/water (5 mmol/L sodium phosphate, 5 mmol/L tetraethylammonium chloride, pH 6.93) at a flow rate of 0.9 mL/min. These conditions afforded PMEA a retention time of 5.8 min; however, the total run time was set at 20 min to ensure the elimination of all endogenous materials between sample injections. The fluorometer was set at an excitation wavelength of 236 nm, with an emission wavelength of 420 nm. The HPLC method had a wide linear range of 2.8 to 91.9 μg/mL in plasma, each 10 μL of supernatant was injected onto a C18 column (3.5 μm, 4.6 mm × 75 mm; Zorbax SB) using a mobile phase of 40% acetonitrile/30 mmol/L KH2PO4 at a flow rate of 0.9 mL/min, and detected at a wavelength of 244 nm. This method had a linear range of 2.8 to 91.9 μg/mL, and was accurate and precise with percentages of coefficients of variation of <15%.

For the analysis of probenecid in plasma, aliquots of 10 μL of mouse plasma were mixed with 100 μL of methanol and 20 μL of acetonitrile by vortexing for 1 min. After centrifugation at 15,000 rpm for 5 min, a 20 μL aliquot of the supernatant was injected onto the HPLC system. Probenecid was eluted at 4 min on a C18 column (3.5 μm, 4.6 mm × 75 mm; Zorbax SB) using a mobile phase of 46% acetonitrile/30 mmol/L KH2PO4 at a flow rate of 0.9 mL/min, and detected at a wavelength of 214 nm. This method had a linear range of 0.5 to 11.9 μg/mL, and was accurate and precise with percentages of coefficients of variation of <15%.

**Pharmacokinetic analyses.** A linear two-compartment open pharmacokinetic model was fit to each individual mouse PMEA plasma concentration-time profile using maximum likelihood optimization with a fractional SD variance model as implemented in the SAAM II program (20). Each best-fit model yielded a set of model variables that were used to calculate primary pharmacokinetic variables of total systemic clearance, volume of distribution at steady-state, and the elimination half-life of PMEA. Renal clearance was determined by dividing the total amount of PMEA excreted in the urine in 6 h by the area under the plasma concentration-time curve over the same time period. For brain PMEA experiments, individual steady-state brain/plasma PMEA concentration ratios were calculated.

**Statistical analyses.** Body weights and WBC counts were analyzed using the Student’s t test. Mouse survival data were modeled using logistic regression. The model included PMEA dose, mouse type, and their interaction. ANOVA was completed to determine if there were statistical differences (P < 0.05) between the pharmacokinetic variables as a function of dose and species and probenecid coadministration. Differences in brain/plasma PMEA concentrations were analyzed using a one-way ANOVA.

**Results**

**Generation of Mrp4−/− mice.** To examine the function of Mrp4, an Mrp4-null mouse was generated by homologous recombination in ES cells, using a vector that targeted sequences encoding amino acids 77 to 101 (Fig. 1A–C). Immunoblot analyses of several tissues from Mrp4−/− mice, including kidney, spleen, small intestine, and colon confirmed the absence of Mrp4 protein (Fig. 1D). Mrp4-null mice appeared normal with respect to fertility, appearance, behavior, serum chemistry, and hematologic variables, and histopathologic analysis did not reveal any abnormalities (data not shown).

**Mrp4−/− mice are hypersensitive to PMEA.** To investigate the in vivo pharmacologic functions of Mrp4, wild-type and Mrp4-null mice were challenged with the nucleotide analogue PMEA, an agent with antiproliferative and antiviral activity that is a well characterized component of the MRP4 drug resistance profile (10, 11, 21). Mrp4−/− mice exhibited increased sensitivity to this agent, as indicated by the clear separation of the dose-response curves (Fig. 2A). The minimal toxic dose for Mrp4−/− mice was 0.5 g/kg, or 5-fold lower than that of wild-type mice (2.5 g/kg). The dose at which 50% of Mrp4-null animals died was ~50% of the corresponding dose for wild-type mice. Notably, at 2 g/kg, all
Mrp4-null mice died, whereas all wild-type mice survived. The increased sensitivity of Mrp4–/– mice treated at this dose was reflected by their inability to maintain body weight, which was evident as early as 24 h after treatment (Fig. 2B). By day 5, Mrp4–/– mice lost >20% of body weight. By comparison, wild-type mice lost <5% of body weight at this time point. In addition, the peripheral white blood depression was prolonged in Mrp4-null mice, with the nadir reached at day 4, whereas with wild-type mice, the nadir was reached 1 to 2 days after treatment (Fig. 2C).

**Mrp4 protects bone marrow, thymus, spleen, and intestine.** Histopathologic analysis of wild-type and Mrp4–/– mice treated with PMEA revealed striking changes in many tissues of Mrp4–/– mice, whereas in all cases, the corresponding tissues of wild-type mice appeared completely normal (Figs. 3 and 4). Intense aplasia was evident in the bone marrow of Mrp4-null animals (Fig. 3A). Mrp4-null mice also had smaller spleens in which the lymphoid follicles were diminished in number and size (Fig. 3B). In addition, erythroid and myeloid cell populations in the red pulp were decreased and replaced by stromal cells (seen at higher power magnification; data not shown). In Mrp4–/– mice, there was almost complete lymphocyte depopulation in the cortical regions of the thymus (Fig. 3C). Marked toxicity was also apparent in the gastrointestinal tract of Mrp4-null mice (Fig. 4). Acute mucositis destroyed the epithelium of the forestomach, leaving only the horny layer and denuded subepithelial tissue in which there were inflammatory cells and edematous changes (Fig. 4A). In Mrp4-null animals, the small intestinal villi were decreased in size, and there were prominent inflammatory infiltrates in the submucosa (Fig. 4B). In addition, the basal portions of the glands were dilated. Mrp4–/– animals had severe atrophic toxic colitis characterized by decreased thickness of colonic mucosa, loss of glands, and the presence of inflammatory infiltrates (Fig. 4C). Mucous microcrypts were also present.

**Analysis of systemic PMEA pharmacokinetics in Mrp4–/– mice.** PMEA is predominately eliminated unchanged in the urine of humans and mice (22, 23). The reported localization of Mrp4 to apical membranes in proximal renal tubules (15, 16), in
combination with its facility for transporting this compound, suggested that Mrp4 might be involved in the renal elimination of PMEA. If this were the case, Mrp4-null mice would be expected to have higher serum levels of PMEA, and the observed chemosensitivity phenotype of Mrp4<sup>−/−</sup> mice could be attributable to systemic pharmacokinetic alterations as opposed to increased inherent tissue sensitivity resulting from loss of a cellular resistance factor. A pharmacokinetic analysis of PMEA was therefore conducted to ascertain whether Mrp4 is involved in the renal elimination of PMEA and whether it affects PMEA serum levels. These experiments showed that neither urinary clearance nor plasma concentrations of PMEA were altered in Mrp4<sup>−/−</sup> mice.

When measured at two different PMEA concentrations, the plasma-time profiles of wild-type and Mrp4<sup>−/−</sup> mice were indistinguishable (Fig. 5A; profile for 5 mg/kg not shown), and significant differences were not observed in overall clearance or urinary clearance in wild-type and Mrp4<sup>−/−</sup> mice (Table 1). Probenecid, a general inhibitor of organic anion transporters including MRP4 (15), significantly decreased urinary clearance in wild-type mice, with a corresponding decrease in total clearance and an increase in serum PMEA levels (Fig. 5B; Table 1). However, genetic ablation of Mrp4 in combination with probenecid treatment did not decrease total clearance or urinary clearance of PMEA beyond the effect of probenecid on wild-type mice (Table 1), demonstrating that whereas PMEA urinary elimination is mediated by organic anion transporters, Mrp4 is not a rate-limiting component of this probenecid-sensitive system.

Mrp4 restricts PMEA penetration into the brain. Next, the ability of Mrp4, which was reported to be expressed on the apical surfaces of brain capillaries (16), to affect the penetration of PMEA into the brain was analyzed. Under steady-state conditions, Mrp4-null mice had 1.8-fold higher brain/plasma ratios of PMEA compared with wild-type mice (Fig. 5C), implicating Mrp4 in brain accumulation of PMEA under conditions analogous to those of a multiple-dose administration schedule that would be used clinically.

Discussion

Investigations of cell lines that overexpress MRP4 have established that the pump is able to confer resistance to a variety of anticancer and antiviral agents. However, the facility of MRP4 to confer in vivo resistance has not been determined. To address this question, we used an approach involving the analysis of toxicity and pharmacokinetics in Mrp4-null mice treated with PMEA. We found that Mrp4<sup>−/−</sup> mice do not exhibit obvious abnormalities, but they are more susceptible than wild-type mice to challenge with PMEA. This finding, in combination with the absence of alterations in plasma PMEA levels, provides the first direct evidence that Mrp4 functions as an in vivo resistance factor. The striking pattern of toxicity in hematopoietic, lymphopoietic, and gastrointestinal sites is consistent with the interpretation that increased lethality in Mrp4-null mice is attributable to neutropenia that may be exacerbated by the breakdown of the gastrointestinal barrier to gut flora. Acute toxicity in Mrp4<sup>−/−</sup> mice was confined to proliferating tissues. This toxicity pattern likely reflects the known predilection of cytotoxic agents to affect rapidly dividing tissues, as opposed to being attributable to the restriction of Mrp4 expression to affected tissues. This notion is supported, respectively, by the ability of PMEA to inhibit DNA synthesis (21), and by the absence of apparent toxicity in kidney, a tissue in which Mrp4 is expressed at levels that are at least comparable to those in tissues that are susceptible to damage, such as spleen and gut (Fig. 1D). The wide distribution of MRP4 in the body suggests that the pump is likely to affect the levels of nucleotide analogues in tissues in which we did not observe increased toxicity. Our results also suggest that Mrp4 may affect in vivo sensitivity to other nucleoside-based analogues that are part of the resistance profile of the pump, such as the nucleobase and nucleoside analogues 6-mercaptopurine, 6-thioguanine, and ganciclovir. However, it should be borne in mind that the pump's in vivo activity towards these agents may not be entirely analogous to PMEA because, in contrast with PMEA, which is an amphipathic anion that is a direct substrate of Mrp4, is not a rate-limiting factor. The pump's in vivo activity towards these agents may not be entirely analogous to PMEA because, in contrast with PMEA, which is an amphipathic anion that is a direct substrate of Mrp4, is not a rate-limiting factor.
to transport by MRP4, as opposed to their unchanged parent compounds (13). In addition, there may also be considerations that pertain to the transport kinetics of the pump. Because PMEA is relatively nontoxic, high concentrations were required to elicit toxicity in our experiments, and it is possible that the effects of the pump may be different in experiments employing lower concentrations of more toxic agents. Additional experiments on Mrp4−/− mice should help to clarify whether there are differences in how the pump affects various agents in vivo. Finally, our results suggest that previously reported single nucleotide polymorphisms in the ABCC4 gene might be of pharmacologic significance (24).

Renal disposition of PMEA is of particular interest because treatment with this agent is associated with delayed nephrotoxicity (25). Renal elimination of organic anions such as PMEA involves the concerted action of basolateral transporters that mediate uptake into renal tubule cells from the peritubular circulation and apical transporters that mediate extrusion into the urine. Although Mrp4 is expressed at the apical surfaces of kidney proximal tubules, and PMEA is predominately eliminated in the urine, reduced renal clearance of PMEA was not observed in Mrp4−/− mice. This finding, in combination with the susceptibility of renal PMEA elimination to inhibition by probenecid, suggests that the rate-limiting components of the probenecid-sensitive system responsible for renal excretion of PMEA may be composed of basolateral organic anion transporters that are capable of transporting PMEA, such as OAT1 (26), and possibly apical organic anion transporters other than Mrp4. Although renal clearance of PMEA was not affected in Mrp4-null mice, it will be of interest to determine whether these mice have increased susceptibility to delayed renal toxicity under conditions of chronic administration.

The finding that Mrp4−/− mice have increased concentrations of PMEA in the brain extends the capabilities of Mrp4 that were inferred from a study showing that Mrp4 restricts brain accumulation of the anticancer agent topotecan (16). Our experiments suggest that, in addition to certain natural product agents, the pump may also affect the accumulation of nucleotide analogues in the brain by functioning as a component of the blood-brain barrier. Given that the antiretroviral agent PMPA (tenofovir), which structurally resembles PMEA, and 3′-azido-3′-deoxythymidine, are likely to be transported by MRP4 (11), this finding may be relevant with respect to the treatment of AIDS. With the success of highly active antiretroviral therapy for the treatment of systemic HIV infection, the inability to eradicate latent or slowly replicating HIV in the central nervous system has emerged as a growing concern (27). Our results, in combination with the role that P-glycoprotein plays at the blood-brain barrier to limit the penetration of protease inhibitors (28), suggests that the levels in the brain of two arms of highly active antiretroviral treatments may be limited by efflux pumps. Similarly, it is possible that MRP4 may also affect the transport of cytomegalovirus retinitis with ganciclovir in patients with AIDS or other forms of immunocompromise.

### Table 1. Analysis of PMEA pharmacokinetics in wild-type and Mrp4−/− mice

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NOTE: PMEA pharmacokinetic variables in wild-type and Mrp4-null mice were determined at 5 and 25 mg/kg (see Materials and Methods for details).

*The values are means ± SD except for T_{1/2} values which are expressed as the harmonic mean ± pseudo SD (n = 6–8 in each group).

The effect of probenecid on PMEA pharmacokinetics was analyzed at a steady-state plasma probenecid concentration of ~50 μg/mL, which was achieved by a dosing regimen consisting of 20 mg/kg probenecid as an i.v. bolus followed by a constant rate infusion of 20 mg/kg/h for 8 h.

Significantly different (P < 0.05, ANOVA) compared with corresponding PMEA-alone treatment groups.

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