187Lu-EC0800 Combined with the Antifolate Pemetrexed:

Preclinical Pilot Study of Folate Receptor Targeted Radionuclide Tumor Therapy

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Abstract (max. 250 words)

Targeted radionuclide therapy has shown impressive results for the palliative treatment of several types of cancer diseases. The folate receptor (FR) has been identified specifically associated with a variety of frequent tumor types. Therefore, it is an attractive target for the development of new radionuclide therapies using folate-based radioconjugates. Previously, we found that pemetrexed (PMX) has a favorable effect to reduce undesired renal uptake of radiofolates. Moreover, PMX also acts as a chemotherapeutic and radiosensitizing agent on tumors. Thus, the aim of our study was to investigate the combined application of PMX and the therapeutic radiofolate, $^{177}$Lu-EC0800. Determination of the combination index (CI) revealed a synergistic inhibitory effect of $^{177}$Lu-EC0800 and PMX on the viability of FR positive cervical (KB) and ovarian (IGROV-1) cancer cells in vitro (CI < 0.8). In an in vivo study, tumor bearing mice were treated with $^{177}$Lu-EC0800 (20 MBq) and a subtherapeutic (0.4 mg) or therapeutic amount (1.6 mg) of PMX. Application of $^{177}$Lu-EC0800 with PMX$_{ther}$ resulted in a 2-4-fold enhanced tumor growth delay and a prolonged survival of KB and IGROV-1 tumor-bearing mice, as compared to the combination with PMX$_{subther}$ or untreated control mice. PMX$_{subther}$ protected the kidneys from undesired side effects of $^{177}$Lu-EC0800 (20 MBq) by reducing the absorbed radiation dose. Intact kidney function was demonstrated by determination of plasma parameters and quantitative SPECT using $^{99m}$Tc-DMSA. Our results confirmed the anticipated dual role of PMX. Its unique features resulted in an improved antitumor effect of folate-based radionuclide therapy, and prevented undesired radio-nephrotoxicity.
Introduction

Targeted radionuclide therapy has shown impressive results for the palliative treatment of several cancer diseases. It is based on the use of particle-emitting radioisotopes (e.g. $^{177}$Lu $^{90}$Y, $^{131}$I) in conjunction with tumor-targeted biomolecules (e.g. peptides, antibodies) (1). A prominent example of a successfully employed radiopharmaceutical in clinical routine are somatostatin-based radiopeptides (e.g. $^{177}$Lu-DOTATATE, $^{90}$Y-DOTATOC) for the treatment of neuroendocrine tumors (2). Moreover, radiolabeled antibodies such as $^{90}$Y-Ibritumomab (Zevalin™) and $^{131}$I-tositumomab (Bexxar™) are approved for the treatment of non-Hodgkins lymphoma (3).

The development of new targeting strategies for the treatment of further tumor types is of high interest and would have a critical impact on the future management of these cancer diseases. In this respect the folate receptor (FR) is an attractive target as it has been identified specifically associated with a variety of cancer types, such as ovarian, endometrial, lung, brain, breast and colorectal cancer (4, 5). The vitamin folic acid has been used as a targeting ligand because it binds to the FR with high affinity followed by endocytotic internalization of the therapeutic payload into cancer cells (6). While folic acid conjugates of highly toxic chemotherapeutics have been successfully employed in clinical trials (7, 8), application of therapeutic folic acid radioconjugates is currently being developed in preclinical studies.

Substantial expression of the FR in the proximal tubule cells of the kidneys results in commonly high and specific renal uptake of folate based radioconjugates (9, 10). As a consequence there is an inherent risk of damage to the kidneys by particle-radiation. However, we have shown in several preclinical studies that administration of the antifolate pemetrexed (PMX) resulted in a tremendous reduction of the radiofolate’s retention in the kidneys while accumulation in tumor xenografts remained unaffected (11-13). The exact mechanism of this interaction is still not completely clear. However, in numerous preclinical studies we observed an interrelation between the kidney reducing effect and the time point of pre-injected PMX (14) as well as the molar...
amount of PMX and the folate radioconjugate, respectively (15). On the other hand, the reduced kidney uptake of radiofolates was not a result of PMX’s antifolate activity as the effect was maintained even if PMX was applied in combination with the antidote thymidine (16). These facts suggest that PMX’s kidney reducing effect is based on a competition among PMX and the folate radioconjugate for FR binding sites in the kidneys.

PMX is a multi-targeted antifolate which is clinically approved for the treatment of pleural mesothelioma and non-small cell lung cancer in combination with cisplatin (17-19). Moreover, PMX is currently being tested in a number of clinical trials for the treatment of several other cancer types (reviewed in (20)), among those is also ovarian cancer (21). It has been used in combinations with gemcitabine, tyrosine kinase inhibitors, antibodies or even external radiation are used (22-24). A combination of PMX with external radiotherapy was based on the observation that PMX acts as a radiosensitizing agent in variable types of cancer cells in vitro and in vivo (25-28).

We hypothesized that PMX would have a dual role if it was combined with therapeutic folate radioconjugates. Firstly, it was expected to prevent radionephropathy by reducing the absorbed radiation dose of folate radioconjugates in the kidneys. Secondly, PMX was believed to enhance the therapeutic efficacy of folate-based radionuclide tumor therapy by its action as a chemotherapeutic and radiosensitizing agent.

The goal of this study was to demonstrate the anticipated dual role of PMX. For this purpose we employed an established DOTA-folate conjugate (EC0800 (29)) which was radiolabeled with $^{177}$Lu ($T_{1/2} = 6.7$ d, $E_{av}^{(\beta^-)}$: 134 keV, $E^{(\gamma)}$: 113 keV, 208 keV). $^{177}$Lu-EC0800 was applied in combination with PMX using two FR-positive human cancer cells (KB and IGROV-1) in vitro and as human tumor xenografts in athymic nude mice. Moreover, the protective effect of PMX on the kidneys to reduce the absorbed radiation dose was investigated in non-tumor bearing mice.
Material and Methods

Preparation of $^{177}$Lu-EC0800. The DOTA-folate conjugate (EC0800 (29)) was kindly provided by Endocyte Inc. (Supplementary Fig. S1). The radiosynthesis of $^{177}$Lu-EC0800 and the evaluation of its stability were carried out as previously reported (Supplementary Methods) (30). It was shown that $^{177}$Lu-EC0800 was stable (> 95%) over at least 24 h in PBS and human blood plasma.

Cell culture. KB cells (human cervical carcinoma cells, ACC-136), and PC-3 (human prostate carcinoma cells, ACC-465) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). IGROV-1 cells (human ovarian carcinoma cells) were a kind gift from Dr. Gerrit Jansen (Department of Rheumatology, VU University Medical Center, Amsterdam, the Netherlands). All three cell lines were authenticated by DMSZ based on DNA profiling. KB and IGROV-1 cells are known to express the FR (31, 32), while FR negative PC-3 cells (33) were used as a negative control. PC-3 cells were cultured in RPMI 1640 medium (Amimed, Bioconcept, Switzerland) whereas KB and IGROV-1 cells were cultured in folate-free RPMI medium referred to as FFRPMI (without folic acid, vitamin B$_{12}$ and phenol red; Cell Culture Technologies GmbH, Gravesano/Lugano, Switzerland). Cell culture media were supplemented with 10% fetal calf serum, L-glutamine and antibiotics (penicillin/streptomycin/fungizone). Routine culture treatment was performed twice a week. Uptake and internalization studies of $^{177}$Lu-EC0800 as well as clonogenic and MTT assays were performed as previously reported in the literature in detail in the Supplementary Methods (29, 34).

MTT assay. Cell viability was assessed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (35). In brief, KB, IGROV-1 and PC-3 cells were
treated with 200 μL FFRPMI medium (without supplements) containing $^{177}$Lu-EC0800 (0.001-5.0 MBq/mL) and/or PMX (0.01 - 100 μM; pemetrexed, Alimta™, LY231514; Eli Lilly, Bad Homburg, Germany, Supplementary Fig. S1). The control cells were incubated with FFRPMI medium only. After 4 h incubation at 37°C, the cells were washed once with 200 μL PBS followed by addition of FFRPMI or RPMI medium (with supplements). Cells were then allowed to grow for 4 days at 37°C without changing medium. Analysis of the viability was performed as previously reported using an MTT reagent and a microplate reader (Victor X3, Perkin Elmer) (36). To quantify cell viability, the absorbance of the test samples was expressed as percentage of the absorbance of the control cell samples (= 100%). Dose response curves were analyzed using the software GraphPad Prism (version 4.0). Inhibition of cell viability was expressed as the half-maximal inhibitory concentration of PMX (IC$_{50}$ in mmol/L) and as the half-maximal inhibitory activity concentration of $^{177}$Lu-EC0800 (IAC$_{50}$ in MBq/mL) by measuring dose-response curves of PMX and $^{177}$Lu-EC0800. The dose-response curves were used to determine the combination index (CI) according to Chou et al. (Supplementary Methods) (37).

**Animal studies.** The in vivo experiments were approved by the local veterinarian department and conducted in accordance with the Swiss law of animal protection. Female athymic nude mice (six to eight-week-old CD-1 Foxn-1/nu, Charles River Laboratories, Sulzfeld, Germany) were fed with a folate-deficient rodent diet (Harlan Laboratories, U.S.) starting 5 d prior to tumor cell inoculation (38). For therapy experiments endpoint criteria were defined as (i) a tumor volume $>$ 1000 mm$^3$, (ii) body weight loss of $>$ 15%, (iii) active ulceration of the tumor xenograft or (iv) abnormal behavior of the mice and signs of unease.

**Biodistribution study and dosimetric calculations.** Biodistribution studies over 72 h were performed as previously reported (Supplementary Methods, Table S1) (29). These data sets were employed to estimate the equivalent absorbed radiation dose to the tumor xenografts and kidneys.
upon injection of $^{177}$Lu-EC0800 (Supplementary Methods). Based on the biodistribution results the accumulation of radioactivity in KB and IGROV-1 tumors was taken as equal. For estimation of the kidney dose, it was assumed that in the case of pre-injected PMX kidney uptake was reduced to 25% of control values.

Biodistribution studies performed with $^3$H-PMX are reported in the Supplementary Methods.

**Investigation of potential radiotoxicity.** Groups of 6 mice were injected with only PBS (group A), with $^{177}$Lu-EC0800 (20 MBq, 1 nmol; group B) or with $^{177}$Lu-EC0800 (20 MBq, 1 nmol) and PMXsubther (0.4 mg; group C). From day 21 after start of the therapy the animals were fed with a standard rodent diet. For studying plasma parameters, blood was taken from the sublingual vein collected in heparinized vials at day 50, 130 and 180 or before euthanasia. Blood plasma parameters such as creatinine, blood urea nitrogen, alkaline phosphatase and total bilirubin were determined using a Fuji Dri-Chem 4000i analyzer (Polymed Medical Center AG, Switzerland). Quantitative SPECT to determine renal uptake of $^{99m}$Tc-DMSA in mice was performed in week 3, 15 and 23 of the experiment.

SPECT experiments were performed with a 4-head multiplexing multi-pinhole camera (NanoSPECT/CT, Bioscan Inc. U.S.) using collimators of 4 x 9 holes of a diameter of 1.4 mm. Dimercaptosuccinic acid (DMSA)-kits (TechneScan™) were purchased from Mallinckrodt Inc. The $^{99m}$Tc-radiolabeling was performed according to the instructions of the manufacturer using $^{99m}$Tc-pertechnetate which was freshly eluted from a $^{99m}$Tc-generator (Mallinckrodt Inc.). SPECT images of the kidneys (30 mm scan length) were acquired 2 h after injection of $^{99m}$Tc-DMSA (~30 MBq/mouse). After acquisition, SPECT data were reconstructed iteratively with HiSPECT software (version 1.4.3049, Scivis GmbH) using a $\gamma$-energy of 140 keV for $^{99m}$Tc. Quantification of renal uptake of $^{99m}$Tc-DMSA was carried out as previously reported using InVivoScope post-processing software (version 2.0, Bioscan, Inc.) (39).
**Therapy studies in tumor bearing mice.** Twenty mice were inoculated with KB tumor cells (4.5 x 10^6 in 100 μL PBS, model I) and another 20 mice were inoculated with IGROV-1 tumor cells (6.0 x 10^6 in 100 μL PBS, model II). At the start of the therapy four days later, the average tumor size reached a volume of ~65 mm³ (KB xenografts) and ~40 mm³ (IGROV-1 xenografts). For each study, 4 groups of 5 mice were injected with only PBS (group A), with 2 x 0.8 mg PMX (i.e. PMX_ther, group B), with 1 x 20 MBq ^177^Lu-EC0800 combined with 1 x 0.4 mg PMX (i.e. PMX_subther, group C) and with 1 x 20 MBq ^177^Lu-EC0800 combined with 2 x 0.8 mg PMX (i.e. PMX_ther, group D).

Body weights and tumor volumes were measured three times a week. The tumor volume (V) was determined by measuring two perpendicular diameters with a digital caliper and calculated by using the formula \( V = \left[ \frac{0.5 \times (L \times W^2)}{2} \right] \), where L is the length of the tumor (large diameter) and W is the width (small diameter) (40). The values were indicated as the average of the individual relative tumor volumes (\( RTV = \frac{V_x}{V_0}; V_x = \text{volume at day } x, V_0 = \text{volume at day } 0 \)) and of individual relative body weight (\( RBW = \frac{W_x}{W_0}; W_x = \text{weight at day } x, W_0 = \text{weight at day } 0 \)).

The therapeutic efficacy was expressed as the percentage of tumor growth inhibition (\( \%\text{TGI} = 100 - (T/C) \times 100 \)), where T is the mean RTV of the treated mice and C is the mean RTV of untreated control mice at day 17 when the first mouse in the control group was euthanized.

Tumor growth delay (TGD) was calculated for the time which passed until the RTVs reached 4-fold the value of day 0. The tumor growth delay index (TGDI) was calculated as the ratio of TGDs among treated animals and control animals (41).

**Statistical analysis.** Statistics was performed by using a t-test (*Microsoft Excel* software). All analyses were two-tailed and considered as type 3 (two sample unequal variance). A p-value of <0.05 was considered as statistically significant.
Results

Cancer cell lines. The human cervical KB cancer cell line is a subclone of HeLa cells (42), known to express the FR at high very levels. IGROV-1 cells are human ovarian cancer cells which express the FR at a somewhat lower level than KB cells (12, 31, 36). The human prostate cancer cell line PC-3 does not express the FR and was used as a negative control. These facts were confirmed in uptake/internalization studies of $^{177}$Lu-EC0800 (Supplementary Fig. S2).

Cell survival upon exposure to $^{177}$Lu-EC0800. Clonogenic assays revealed plating efficiencies of 11%, 14%, 18% and 16%, for KB, IGROV-1 and PC-3 cells. At a radioactivity concentration of 1.0 MBq/mL $^{177}$Lu-EC0800 (16 nmol/L) with a 4 h exposure, the survival fraction (SF) of KB and IGROV-1 cells was 0.12 and 0.43, respectively. The SF of FR-negative PC-3 cells was still 0.98, even after the treatment with a 5-fold higher radioactivity concentration. Reduction of cell survival was completely suppressed by pre-incubating FR-positive tumor cells with excess folic acid to block FRs (Fig. 1A, Supplementary Fig. S3).

Cell viability upon exposure of $^{177}$Lu-EC0800 combined with PMX. MTT assays were performed to determine IAC$_{50}$ and IC$_{50}$ values of $^{177}$Lu-EC0800 and PMX. The inhibition of cell viability was found to be dependent on the concentration of PMX in all cell lines. The IC$_{50}$ value of PMX amounted to $1.22 \pm 0.13 \mu$mol/L and $0.93 \pm 0.17 \mu$mol/L for KB and IGROV-1 cells, respectively. The IAC$_{50}$-values of $^{177}$Lu-EC0800 revealed activity concentrations of $0.054 \pm 0.004$ MBq/mL for KB and $0.83 \pm 0.083$ MBq/mL for IGROV-1 tumor cells (Figure 1B-1D).

Combination index (CI). The interactions between $^{177}$Lu-EC0800 and PMX were calculated according to the results obtained with KB and IGROV-1 cells which were exposed to $^{177}$Lu-EC0800 and PMX as single agents or simultaneously. The concentrations of the test agents, alone and in combination, required to reduce cell viability to 55% and 70% of controls were
determined. All calculations revealed values of the CI below 0.8 indicating a synergistic effect between $^{177}$Lu-EC0800 and PMX (Supplementary Table S2).

**Biodistribution studies.** Biodistribution studies in KB and IGROV-1 tumor bearing mice showed a relatively high uptake of $^{177}$Lu-EC0800 in tumor xenografts (KB: 5.94 ± 1.20% ID/g and IGROV-1: 6.58 ± 1.50% ID/g; 4 h p.i.), and a ~10-fold higher accumulation in the FR-positive kidneys. PMX treatment reduced renal uptake of $^{177}$Lu-EC0800 up to 7-fold, while simultaneously allowing for greater tumor uptake (Table 1). Dosimetric estimation revealed a dose of 0.38 Gy/MBq to the tumor xenografts and 4.84 Gy/MBq to the kidneys if $^{177}$Lu-EC0800 was applied as a single agent. Under the assumption of a 4-fold reduced renal uptake of $^{177}$Lu-EC0800 in combination with PMX, the kidney dose was reduced to 1.21 Gy/MBq while the tumor dose remained unaffected (Supplementary Methods, Supplementary Fig. S4).

Short after injection of $^3$H-PMX the uptake in the kidneys was relatively high (~2.7% ID/g, 1 h p.i.) but a significant wash-out was observed within the following hour (~0.8% ID/g, 2 h p.i.). In the tumor tissue accumulation of $^3$H-PMX was low (~1.1% ID/g, 1 h p.i.) but more constant (0.6% ID/g, 2 h p.i.) over time. (Supplementary Methods, Supplementary Fig. S5).

**Investigation of potential radiotoxicity.** In a separate study radiotoxicity of $^{177}$Lu-EC0800 and the kidney protective effect of a subtherapeutic amount of PMX (0.4 mg) were investigated. Non-tumor bearing nude mice were monitored over 6 months. The kidney dose of $^{177}$Lu-EC0800 (20 MBq/mouse) was ~97 Gy (group B). If PMX subther was pre-injected, the kidney dose was significantly reduced to ~24 Gy (group C). At day 50 of the study, plasma parameters of treated mice (groups B/C) were in the same range as those of control mice (group A). However, at day 130 levels of blood urea nitrogen, alkaline phosphatase and total bilirubin from mice treated with $^{177}$Lu-EC0800 (group B) differed significantly from those of control mice (group A). The values obtained from mice of group C were in the same range as the values from mice of group A.
Determination of blood plasma parameters at day 180 showed the same result as found at day 130 (Table 2).

The extent of accumulated $^{99m}$Tc-DMSA in the kidneys is a measure for tubular function (43). It has previously been used as a valuable in vivo tool for monitoring kidney function during radionuclide therapy (39). In week 3 baseline measurements of $^{99m}$Tc-DMSA uptake in the kidneys showed no significant difference among group B and group C from control mice of group A (Fig. 2A). However, in week 15 the average % ID per kidney of mice treated with $^{177}$Lu-EC0800 only (group B: $4.37 \pm 2.6 \%$ ID/kidney, 2 h p.i., $p < 0.005$) was significantly lower than in control mice (group A: $11.74 \pm 0.7 \%$ ID/kidney, 2 h p.i.). Renal uptake of $^{99m}$Tc-DMSA in mice which received PMXsubther in addition to $^{177}$Lu-EC0800 was comparable (group C: $10.58 \pm 1.8 \%$ ID/kidney, 2 h p.i., $p = 0.2$) with the value obtained from control animals (Fig. 2B). In week 23 of follow-up, renal accumulation of $^{99m}$Tc-DMSA in mice of group B had dropped to $1.38 \pm 0.16 \%$ ID/kidney 2 h p.i. ($p < 0.005$), whereas in group C the uptake was still in same range as found for control animals of group A ($p = 0.6$) (Fig. 2C).

Constant body weight loss was observed in mice treated with $^{177}$Lu-EC0800 (group B) from about day 70 and thereafter. In all cases of group B the endpoint criterion which required euthanasia was reached before day 180. However, mice which had received PMXsubther prior to the injection of $^{177}$Lu-EC0800 (group C) showed body weight gain similar to the untreated controls (group A).

**In vivo tumor therapy studies.** The injection protocol of the therapy study with KB and IGROV-1 tumor bearing mice is shown in Figure 3A. PMX was applied either in a subtherapeutic dose of 0.4 mg (PMXsubther) or at a therapeutic dose of 2 x 0.8 mg (PMXther) corresponding to 80% of the maximal tolerated dose (MTD). The MTD of PMX was previously determined in mice under the experimental conditions of a folate-free diet and revealed a dose of 2 x 1 mg per mouse (body weight ~ 25 g) with a time lag of one week (Supplementary Methods, Supplementary Fig. S6) (16).
In both tumor models (I and II) constant tumor growth was observed in control mice (group A) where the first mouse reached the endpoint criterion at day 17. For model I the average RTV in mice treated with PMXther (group B: 9.8 ± 4.1; p = 0.90) and in mice treated with $^{177}$Lu-EC0800 and PMXsubther (group C: 8.3 ± 3.5, p = 0.21) were not significantly different from the average RTV of control mice (group A: 10.0 ± 2.7) at day 17 (Table 3). However, the combined application of $^{177}$Lu-EC0800 with PMXther resulted in a significant decrease of the average RTV (group D: 5.0 ± 2.2; p < 0.0001, Fig. 3B). For tumor model II the average RTV of PMX treated mice (group B: 27.9 ± 11.8; p = 0.73) was not significantly reduced compared to control mice (group A: 30.0 ± 14.9) at the same time point (Table 3). However, a significant reduction of the average RTV was observed in both groups of mice treated with $^{177}$Lu-EC0800 (group C: 7.3 ± 3.9, p = 0.0006; group D: 2.0 ± 1.7, p = 0.0002, Fig. 3C). Monitoring of the body weight revealed slight weight gain over time in model I and a largely constant body weight in model II (Fig. 3D/E). In contrast, pronounced loss of body weight was observed in group B mice of both tumor models (which received PMXther only). Importantly, the average survival time was increased 75-100% in group D mice from both tumor models compared to group A mice (Table 3).

**Discussion**

Combining anticancer therapies is a strategy to broaden the therapeutic index by taking advantage of additive or synergistic antitumor effects and by reducing undesired side effects. The present study addressed the question of whether PMX contributes to the anti-tumor effect of radiofolates and prevents the risk of radionephropathy. In vitro the clonogenic potential of KB and IGROV-1 tumor cells was reduced upon exposure to $^{177}$Lu-EC0800 in a concentration dependent manner (Fig. 1). Moreover it was proven that this effect was specifically FR-mediated. It was more pronounced in KB cells which express the FR at higher levels than IGROV-1 cells, and it was abolished if the cells were co-incubated with excess folic acid to block FR binding of
Inhibition of cell viability through application of $^{177}\text{Lu-EC0800}$ was enhanced if cells were co-incubated with PMX (Fig. 1). Determination of the combination indices at different drug concentrations revealed that $^{177}\text{Lu-EC0800}$ and PMX provided synergistic inhibitory effects on the viability of both tumor cell lines. It was observed that incubation of the cancer cells with PMX resulted in an accumulation of the cells in the G1/S boundary or early S phase as previously reported (Supplementary Fig. S7) (25, 44). On the other hand, exposure of cells to $^{177}\text{Lu-EC0800}$ showed a cell cycle arrest in the G2/M phase, which is a common phenomenon in eukaryotic cells exposed to ionizing radiation (45). However, if the cancer cells were simultaneously exposed to $^{177}\text{Lu-EC0800}$ and PMX the cell cycle arrest in G2/M phase was abrogated. Notably the disruption of the radiation-induced G2-checkpoint by chemotherapeutic agents (e.g. protein kinase inhibitors) was previously shown to sensitize cancer cells to radiation-induced apoptosis and cell death (46-48). This mechanism might also have been responsible for PMX-induced radiosensitization of KB and IGROV-1 cells. An increased apoptotic cell fraction was measured if the cells were treated with $^{177}\text{Lu-EC0800}$ and PMX compared to the application of each of these therapeutics alone (Supplementary Fig. S8).

In vivo the pre-injection of PMX improved the tissue distribution of $^{177}\text{Lu-EC0800}$ dramatically by increasing the tumor-to-kidney ratio (Table 1). In an attempt to understand the underlying mechanism the uptake of $^3\text{H-PMX}$ was determined in KB tumors and kidneys of mice. In the kidneys we found a relatively high uptake 1 h p.i. of $^3\text{H-PMX}$ which was quickly cleared over time (Supplementary Fig. S5). These findings together with the fact that PMX has a high affinity for the FR (49) support our hypothesis of a competition among PMX and $^{177}\text{Lu-EC0800}$ for FR-binding sites in the kidneys if PMX was injected about 1 h prior to the radiofolate. As to what concerns the tumor tissue, uptake of $^3\text{H-PMX}$ was low but retained over time. It has been reported previously that the tumor uptake of PMX is mediated primarily through carriers such as the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT) whereas FRs play
a minor role (50). This may explain the fact that PMX does not compete with the FR-mediated accumulation of $^{177}\text{Lu-EC0800}$ in tumor xenografts.

For the first time we were able to demonstrate in this study that PMX prevents damage to the kidneys by reducing renal accumulation of $^{177}\text{Lu-EC0800}$. Analysis of plasma parameters and the results of SPECT studies using $^{99m}\text{Tc-DMSA}$ consistently confirmed normal kidney function in $^{177}\text{Lu-EC0800}$-treated mice which had received a subtherapeutic amount of PMX. These findings unambiguously confirmed the beneficial role of PMX to prevent radionephropathy of radiofolate therapy.

Based on the in vitro results showing a synergistic effect of PMX and $^{177}\text{Lu-EC0800}$ on the viability of tumor cells, it is likely that the anticancer effect of $^{177}\text{Lu-EC0800}$ would be enhanced by co-application of therapeutic amounts of PMX (2 x 0.8 mg, corresponding to 80% of the MTD (16)). Therapy studies were performed with KB (model I) and IGROV-1 tumor bearing mice (model II) using $^{177}\text{Lu-EC0800}$ combined with either subtherapeutic (0.4 mg/mouse) or therapeutic doses (e.g. 2 x 0.8 mg) of PMX. In both models, tumor growth delay was observed after application of $^{177}\text{Lu-EC0800}$. Application of PMX$_\text{sub}$ alone showed only minor inhibitory effects on growth of these tumor types. However PMX$_\text{ther}$ was able to enhance the anticancer effect of $^{177}\text{Lu-EC0800}$ against both, KB and IGROV-1 tumor xenografts. Also, an increased survival time was achieved if $^{177}\text{Lu-EC0800}$ and PMX were combined compared to the result obtained with each of these agents applied as mono-therapy.

With this study we were able to demonstrate the proposed dual effect of PMX in combination with FR-targeted radionuclide therapy using $^{177}\text{Lu-EC0800}$. On one hand, PMX at subtherapeutic and therapeutic amounts effectively reduced renal uptake of $^{177}\text{Lu-EC0800}$ and therewith prevented long-term radionephropathy (Table 1, Figure 2). On the other hand the application of PMX$_\text{ther}$ enhanced the tumor growth delay induced by $^{177}\text{Lu-EC0800}$. The interplay of the proposed drug combination is absolutely unique. Therefore, we believe that combining PMX with $^{177}\text{Lu-folate}$ therapy warrants further preclinical investigations. Assuming the kidney-reducing
effect of PMX could be confirmed also in man, the combined application of therapeutic radiofolates and PMX has a potential translational impact. Such a therapy protocol would be particularly interesting for the treatment of non-small cell lung cancer which frequently shows FR-overexpression, and for which PMX is an FDA-approved indication.
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References


## Tables

**Table 1**: Biodistribution data 4 h after injection of $^{177}$Lu-EC0800 (3 MBq, 1 nmol) in KB (model I) and IGROV-1 tumor bearing nude mice (model II).

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<tr>
<td>tumor-to-kidney</td>
<td>0.12 ± 0.02</td>
<td>0.86 ± 0.14</td>
<td>1.11 ± 0.16</td>
</tr>
</tbody>
</table>

**177$^{Lu}$-EC0800 (model II)**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>$^{1}$PMX$_{subther}$</th>
<th>$^{2}$PMX$_{ther}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>0.16 ± 0.04</td>
<td>0.08 ± 0.00</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>liver</td>
<td>7.60 ± 2.04</td>
<td>2.75 ± 0.19</td>
<td>2.37 ± 0.68</td>
</tr>
<tr>
<td>kidneys</td>
<td>67.0 ± 13.4</td>
<td>11.4 ± 1.2</td>
<td>9.30 ± 1.96</td>
</tr>
<tr>
<td>tumor</td>
<td>6.58 ± 1.50</td>
<td>9.30 ± 1.96</td>
<td>9.55 ± 0.88</td>
</tr>
<tr>
<td>tumor-to-blood</td>
<td>42.7 ± 7.8</td>
<td>122.7 ± 28.0</td>
<td>127.9 ± 40.6</td>
</tr>
<tr>
<td>tumor-to-liver</td>
<td>0.88 ± 0.16</td>
<td>3.40 ± 0.78</td>
<td>4.32 ± 1.48</td>
</tr>
<tr>
<td>tumor-to-kidney</td>
<td>0.10 ± 0.02</td>
<td>0.81 ± 0.13</td>
<td>1.04 ± 0.54</td>
</tr>
</tbody>
</table>

**Note**: Data are presented as average ± SD (n = 3)

$^{1}$PMX$_{subther}$: 0.4 mg per mouse, injected 1 h prior to $^{177}$Lu-EC0800

$^{2}$ PMX$_{ther}$: 0.8 mg per mouse, injected 1 h prior to $^{177}$Lu-EC0800
Table 2: Plasma parameters of group A (PBS), group B (20 MBq of $^{177}$Lu-EC0800) and group C (20 MBq of $^{177}$Lu-EC0800 and 0.4 mg of PMX): creatinine (CRE), blood urea nitrogen (BUN), alkaline phosphatase (ALP) and total bilirubin (TBIL).

<table>
<thead>
<tr>
<th>Day</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>$^{177}$Lu-EC0800</td>
<td>$^{177}$Lu-EC0800 &amp; PMX&lt;sub&gt;subther&lt;/sub&gt;</td>
</tr>
<tr>
<td>CRE [μmol/L]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>21 ± 3.4</td>
<td>19 ± 2.1</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>130</td>
<td>28 ± 12</td>
<td>58 ± 43</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>180*</td>
<td>19 ± 0.7</td>
<td>114 ± 62*</td>
<td>21 ± 0.7</td>
</tr>
<tr>
<td>BUN [mmol/L]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>8.5 ± 0.9</td>
<td>11.8 ± 2.2*</td>
<td>10.0 ± 1.1</td>
</tr>
<tr>
<td>130</td>
<td>9.7 ± 0.6</td>
<td>35.0 ± 9.4**</td>
<td>8.61 ± 1.1</td>
</tr>
<tr>
<td>180*</td>
<td>8.8 ± 0.6</td>
<td>&gt; 49.98**</td>
<td>9.09 ± 1.4</td>
</tr>
<tr>
<td>ALP [U/l]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>78 ± 10</td>
<td>83 ± 20</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>130</td>
<td>54 ± 16</td>
<td>132 ± 42**</td>
<td>71 ± 18</td>
</tr>
<tr>
<td>180*</td>
<td>58 ± 14</td>
<td>123 ± 55*</td>
<td>67 ± 23</td>
</tr>
<tr>
<td>TBL [μmol/L]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10 ± 1.3</td>
<td>8 ± 1.0</td>
<td>11 ± 1.7</td>
</tr>
<tr>
<td>130</td>
<td>12 ± 1.9</td>
<td>24 ± 11*</td>
<td>12 ± 4.5</td>
</tr>
<tr>
<td>180*</td>
<td>12 ± 3.2</td>
<td>36 ± 1.9**</td>
<td>12 ± 5.0</td>
</tr>
</tbody>
</table>

* > 0.05; ** > 0.005; * group B terminal blood sampling
Table 3: Results of the therapy studies with KB and IGROV-1 tumor bearing mice using $^{177}$Lu-EC0800 and/or PMX.

<table>
<thead>
<tr>
<th>Model I: KB tumor xenografts</th>
<th>Group</th>
<th>PMX [mg]</th>
<th>Radioactivity [MBq]</th>
<th>RTV day 17</th>
<th>TGI [%]</th>
<th>TGDI$_4$</th>
<th>Add. survival time [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>10.0 ± 2.7</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>2 x 0.8 (ther)</td>
<td>-</td>
<td>9.8 ± 4.1</td>
<td>2.1</td>
<td>1.2</td>
<td>+ 22.5%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1 x 0.4 (subther)</td>
<td>1 x 20</td>
<td>8.3 ± 3.5</td>
<td>16.9</td>
<td>1.4</td>
<td>+ 50.0%</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2 x 0.8 (ther)</td>
<td>1 x 20</td>
<td>5.0 ± 2.2</td>
<td>50.4</td>
<td>1.9</td>
<td>+ 75.0%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model II: IGROV-1 tumor xenografts</th>
<th>Group</th>
<th>PMX [mg]</th>
<th>Radioactivity [MBq]</th>
<th>RTV day 17</th>
<th>TGI [%]</th>
<th>TGDI$_4$</th>
<th>Add. survival time [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>30.0 ± 14.9</td>
<td>0.0</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>2 x 0.8 (ther)</td>
<td>-</td>
<td>27.9 ± 11.8</td>
<td>7.1</td>
<td>1.8</td>
<td>+ 0%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1 x 0.4 (subther)</td>
<td>1 x 20</td>
<td>7.3 ± 3.9</td>
<td>75.8</td>
<td>2.6</td>
<td>+ 63.0%</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2 x 0.8 (ther)</td>
<td>1 x 20</td>
<td>2.0 ± 1.7</td>
<td>93.2</td>
<td>4.0</td>
<td>+ 100%</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1:** A, Survival of FR positive KB and IGROV-1 cells and FR negative PC-3 cells upon exposure to $^{177}$Lu-EC0800 (1 MBq/mL; 16 nmol/L) in the presence and absence of excess folic acid (FA). B/C, Dose-response curves of KB and IGROV-1 tumor cells incubated with variable activity concentrations of $^{177}$Lu-EC0800 (indicated as $^{177}$Lu) and PMX.

**Figure 2:** A-C, The average %ID per kidney in week 3, 15 and 23 after injection of $^{99m}$Tc-DMSA in untreated control mice (group A), mice treated with $^{177}$Lu-EC0800 (group B) and mice treated with $^{177}$Lu-EC0800 and PMX<sub>subther</sub> (group C) (** p < 0.005). D, Graphs of the relative body weights of mice from each group over the whole time of 6 months. The lowest tolerable body weight is indicated at a relative value of 0.85. E, SPECT images of kidneys after injection of $^{99m}$Tc-DMSA of one representative mouse of each group in week 23.

**Figure 3:** A, Injection scheme of the in vivo therapy studies performed in KB (model I) and IGROV-1 tumor bearing mice (model II). B, Graphs of the mean relative tumor volumes of KB tumor xenografts (model I). C, Graphs of mean relative tumor volumes of IGROV-1 xenografts (model II). D, Graphs of the mean relative body-weights of KB tumor bearing mice (model I). E, Graphs of the mean relative body-weight of IGROV-1 tumor bearing mice (model II).
Molecular Cancer Therapeutics

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Josefine Reber, Stephanie Haller, Christopher P Leamon, et al.

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