Small Molecule Therapeutics

177Lu-EC0800 Combined with the Antifolate Pemetrexed: Preclinical Pilot Study of Folate Receptor Targeted Radionuclide Tumor Therapy

Josefine Reber¹, Stephanie Haller¹, Christopher P. Leamon², and Cristina Müller¹

Abstract
Targeted radionuclide therapy has shown impressive results for the palliative treatment of several types of cancer diseases. The folate receptor has been identified as 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 in reducing 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 177Lu-EC0800. Determination of the combination index (CI) revealed a synergistic inhibitory effect of 177Lu-EC0800 and PMX on the viability of folate receptor–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 177Lu-EC0800 (20 MBq) and a subtherapeutic (0.4 mg) or therapeutic amount (1.6 mg) of PMX. Application of 177Lu-EC0800 with PMXther resulted in a two- to four-fold enhanced tumor growth delay and a prolonged survival of KB and IGROV-1 tumor-bearing mice, as compared to the combination with PMXsubther or untreated control mice. PMXsubther protected the kidneys from undesired side effects of 177Lu-EC0800 (20 MBq) by reducing the absorbed radiation dose. Intact kidney function was shown by determination of plasma parameters and quantitative single-photon emission computed tomography using 99mTc-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. Mol Cancer Ther; 12(11); 2436–45. ©2013 AACR.

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., 177Lu, 90Y, 131I) in conjunction with tumor-targeted biomolecules (e.g., peptides, antibodies; ref. 1). A prominent example of a successfully used radiopharmaceutical in clinical routine are somatostatin-based radiopeptides (e.g., 177Lu-DOTATATE, 90Y-DOTATOC) for the treatment of neuroendocrine tumors (2). Moreover, radiolabeled antibodies such as 90Y-Ibritumomab (Zevalin) and 131I-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 is an attractive target as it has been identified as specifically associated with a variety of cancer types, such as ovarian, endometrial, lung, brain, breast, and colorectal cancer (4, 5). The vitamin folinic acid has been used as a targeting ligand because it binds to the folate receptor 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 used in clinical trials (7, 8), application of therapeutic folic acid radioconjugates is currently being developed in preclinical studies.

Substantial expression of the folate receptor in the proximal tubule cells of the kidneys results in commonly high and specific renal uptake of folate-based radioligands (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 whereas accumulation in tumor xenografts remained unaffected (11–13). The exact mechanism of this interaction is still not completely clear.

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However, in numerous preclinical studies, we observed an interrelation between the kidney reducing effect and the time point of preinjected PMX ([14] as well as the molar amount of PMX and the folate radioconjugate, respectively ([15]). However, the reduced kidney uptake of folates 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 folate receptor binding sites in the kidneys.

PMX is a multitargeted 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 ref. 20]), among those is also ovarian cancer ([21]). PMX has been used in combinations with gemcitabine, tyrosine kinase inhibitors, antibodies, or even external radiation ([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. First, it was expected to prevent radionephropathy by reducing the absorbed radiation dose of folate radioconjugates in the kidneys. Second, PMX was believed to enhance the therapeutic efficacy of folate-based radiopharmaceuticals by its action as a chemotherapeutic and radiosensitizing agent.

The goal of this study was to show the anticipated dual role of PMX. For this purpose, we used an established DOTA-folate conjugate (EC0800; ref. 29), which was radiolabeled with $^{177}$Lu ($T_{1/2} = 6.7$ days, $E_{av} = 134$ keV, $E_{(l)3} = 113$ keV, 208 keV). $^{177}$Lu-EC0800 was applied in combination with PMX using 2 folate receptor positive human cancer cells (KB and IGROV-1) in vitro and as a chemotherapeutic and radiosensitizing agent.

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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 hours were conducted
as previously reported (Supplementary Methods and
Table S1; ref. 29). These datasets were used 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
preinjected PMX kidney uptake was reduced to 25%
of control values.

Biodistribution studies conducted 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 sub-
lingual vein collected in heparinized vials at day 50, 130,
and 180 or before euthanasia. Blood plasma parameters
such as creatinine, blood urea nitrogen, alkaline phospha-
tase, and total bilirubin were determined using a Fuji Dri-
Chem 4000i analyzer (Polymed Medical Center AG). Quantitative single-photon emission computed tomogra-
y (SPECT) to determine renal uptake of $^{99m}$Tc-DMSA
in mice was conducted in week 3, 15, and 23 of the
experiment.

SPECT experiments were conducted with a 4-head
multiplexing multi-pinhole camera (NanoSPECT/CT;
Bioscan Inc.) using collimators of 4 × 9 holes of a diameter
of 1.4 mm. Dimercaptosuccinic acid (DMSA)-kits (Tech-
tneScan) were purchased from Mallinckrodt Inc. The
$^{99m}$Tc-radiolabeling was conducted according to the
instructions of the manufacturer using $^{99m}$Tc-per-
technetate, which was freshly eluted from a $^{99m}$Tc-gener-
ator (Mallinckrodt Inc.). SPECT images of the kidneys
were acquired 2 hours 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 postprocessing software (version
2.0; Bioscan Inc.; ref. 39).

**Therapy studies in tumor-bearing mice**

Twenty mice were inoculated with KB tumor cells (4.5 ×
10^6 in 100 µL PBS, model I) and another 20 mice were
inoculated with IGROV-1 tumor cells (6.0 × 10^6 in 100 µL
PBS, model II). At the start of the therapy 4 days later, the
average tumor size reached a volume of ~65 mm^3 (KB
xenografts) and ~40 mm^3 (IGROV-1 xenografts). For each
study, 4 groups of 5 mice were injected with only PBS
(group A), with 2 × 0.8 mg PMX (i.e., PMXther, group B),
with 1 × 20 MBq $^{177}$Lu-EC0800 combined with 1 × 0.4 mg
PMX (i.e., PMXsubther group C), and with 1 × 20 MBq
$^{177}$Lu-EC0800 combined with 2 × 0.8 mg PMX (i.e.,
PMXther, group D).

Body weights and tumor volumes were measured 3
times a week. The tumor volume (V) was determined by
measuring 2 perpendicular diameters with a digital caliper
and calculated by using the formula $V = 0.5 \times (L \times W^2)$, where L is the length of the tumor (large
diameter) and W is the width (small diameter; ref. 40).

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the equivalent absorbed radiation dose to the tumor
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PMX (i.e., PMXsubther group C), and with 1 × 20 MBq
$^{177}$Lu-EC0800 combined with 2 × 0.8 mg PMX (i.e.,
PMXther, group D).

Body weights and tumor volumes were measured 3
times a week. The tumor volume (V) was determined by
measuring 2 perpendicular diameters with a digital caliper
and calculated by using the formula $V = 0.5 \times (L \times W^2)$, where L is the length of the tumor (large
diameter) and W is the width (small diameter; ref. 40).

The values were indicated as the average of the individual
relative tumor volumes (RTV = $V_x/V_0$; $V_x$ = volume at
day x, $V_0$ = volume at day 0) and of individual relative
body weight (RBW = $W_x/W_0$; $W_x$ = weight at day x; $W_0$
= weight at day 0). The therapeutic efficacy was expressed
as the percentage of tumor growth inhibition (%TGI = 100
− (T/C) × 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 that passed until the RTVs reached 4-fold the
value of day 0. The tumor growth delay index (TGD) was
calculated as the ratio of TGDs among treated animals
and control animals (41).

**Statistical analysis**

Statistics was conducted by using a t-test (Microsoft Excel software). All analyses were 2-tailed and considered
as type 3 (2 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 folate receptor at
very high levels. IGROV-1 cells are human ovarian cancer
cells that express the folate receptor at a somewhat lower
level than KB cells (12, 31, 36). The human prostate cancer
cell line PC-3 does not express the folate receptor and was
used as a negative control. These facts were confirmed in
uptake/internalization studies of $^{177}$Lu-EC0800 (Supple-
mFig. 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 hours exposure, the survival fraction
of KB and IGROV-1 cells was 0.12 and 0.43, respectively.
The survival fraction of folate receptor 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 preincubating folate
receptor positive tumor cells with excess folic acid to block folate receptors (Fig. 1A; Supplementary Fig. S3). Cell viability upon exposure of $^{177}$Lu-EC0800 combined with PMX

MTT assays were conducted 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 ± 0.13 μmol/L and 0.93 ± 0.17 μmol/L for KB and IGROV-1 cells, respectively. The IAC$_{50}$ values of $^{177}$Lu-EC0800 revealed activity concentrations of 0.054 ± 0.004 MBq/mL for KB and 0.83 ± 0.08 MBq/mL for IGROV-1 tumor cells (Fig. 1B and C).

Combination index

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 hours past injection (p.i).] and a ~10-fold higher accumulation in the folate receptor 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 whereas the tumor dose remained unaffected (Supplementary Methods and Fig. S4).

Short after injection of $^{3}$H-PMX, the uptake in the kidneys was relatively high (~2.7% ID/g, 1 hour p.i.) but a significant washout was observed within the following hour (~0.8% ID/g, 2 hours p.i.). In the tumor tissue accumulation of $^{3}$H-PMX was low (~1.1% ID/g, 1 hour p.i.) but more constant (0.6% ID/g, 2 hours p.i.) over time (Supplementary Methods and 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. Nontumor-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 preinjected, 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 $^{99}$mTc-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 $^{99}$mTc-DMSA uptake in the
kidneys showed no significant difference among groups B and C from control mice of group A (Fig. 2A). However, in week 15 the average % ID per kidney of mice treated with 177Lu-EC0800 only (group B: 4.37 ± 0.26% ID/kidney, 2 hours p.i., P < 0.005) was significantly lower than in control mice (group A: 11.74 ± 0.70% ID/kidney, 2 hours p.i.).

Table 1. Biodistribution data 4 hours after injection of 177Lu-EC0800 (3 MBq, 1 nmol) in KB (model I) and IGROV-1 tumor-bearing nude mice (model II)

<table>
<thead>
<tr>
<th>Model (Group)</th>
<th>PMXsubthera</th>
<th>PMXtherb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.08 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>6.61 ± 1.04</td>
<td>4.28 ± 0.69</td>
</tr>
<tr>
<td>Kidneys</td>
<td>51.0 ± 6.6</td>
<td>9.66 ± 1.14</td>
</tr>
<tr>
<td>Tumor</td>
<td>5.94 ± 1.20</td>
<td>7.83 ± 0.98</td>
</tr>
<tr>
<td>Tumor-to-blood</td>
<td>71.6 ± 19.2</td>
<td>131.1 ± 24.6</td>
</tr>
<tr>
<td>Tumor-to-liver</td>
<td>0.91 ± 0.22</td>
<td>1.88 ± 0.41</td>
</tr>
<tr>
<td>Tumor-to-kidney</td>
<td>0.12 ± 0.02</td>
<td>0.86 ± 0.14</td>
</tr>
</tbody>
</table>

177Lu-EC0800 (model II)

<table>
<thead>
<tr>
<th>Model (Group)</th>
<th>PMXsubthera</th>
<th>PMXtherb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.16 ± 0.04</td>
<td>0.08 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>7.60 ± 2.04</td>
<td>2.75 ± 0.19</td>
</tr>
<tr>
<td>Kidneys</td>
<td>67.0 ± 13.4</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>Tumor</td>
<td>6.58 ± 1.50</td>
<td>9.30 ± 1.96</td>
</tr>
<tr>
<td>Tumor-to-blood</td>
<td>42.7 ± 7.8</td>
<td>122.7 ± 28.0</td>
</tr>
<tr>
<td>Tumor-to-liver</td>
<td>0.88 ± 0.16</td>
<td>3.40 ± 0.78</td>
</tr>
<tr>
<td>Tumor-to-kidney</td>
<td>0.10 ± 0.02</td>
<td>0.81 ± 0.13</td>
</tr>
</tbody>
</table>

NOTE: Data are presented as average ± SD (n = 3).
aPMXsubther: 0.4 mg per mouse, injected 1 hour before 177Lu-EC0800.
bPMXther: 0.8 mg per mouse, injected 1 hour before 177Lu-EC0800.

Table 2. Plasma parameters of group A (PBS), group B (20 MBq of 177Lu-EC0800), and group C (20 MBq of 177Lu-EC0800 and 0.4 mg of PMX)

<table>
<thead>
<tr>
<th>Day</th>
<th>Group A PBS</th>
<th>Group B 177Lu-EC0800</th>
<th>Group C 177Lu-EC0800 and PMXsubther</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE (μmol/L)</td>
<td>50</td>
<td>21 ± 3.4</td>
<td>19 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>28 ± 12</td>
<td>58 ± 43</td>
</tr>
<tr>
<td></td>
<td>180a</td>
<td>19 ± 0.7</td>
<td>114 ± 62b</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>50</td>
<td>8.5 ± 0.9</td>
<td>11.8 ± 2.2b</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>9.7 ± 0.6</td>
<td>35.0 ± 9.4c</td>
</tr>
<tr>
<td></td>
<td>180a</td>
<td>8.8 ± 0.6</td>
<td>&gt;49.98c</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>50</td>
<td>78 ± 10</td>
<td>83 ± 20</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>54 ± 16</td>
<td>132 ± 42c</td>
</tr>
<tr>
<td></td>
<td>180a</td>
<td>58 ± 14</td>
<td>123 ± 55b</td>
</tr>
<tr>
<td>TBIL (μmol/L)</td>
<td>50</td>
<td>10 ± 1.3</td>
<td>8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>12 ± 1.9</td>
<td>24 ± 11b</td>
</tr>
<tr>
<td></td>
<td>180a</td>
<td>12 ± 3.2</td>
<td>36 ± 1.9c</td>
</tr>
</tbody>
</table>

Abbreviations: CRE, creatinine; BUN, blood urea nitrogen; ALP, alkaline phosphatase; TBIL, total bilirubin.
aGroup B terminal blood sampling.
bP < 0.05.
cP < 0.005.
Renal uptake of $^{99m}$Tc-DMSA in mice that received PMX subther in addition to $^{177}$Lu-EC0800 was comparable (group C: 10.58 $\pm$ 1.8% ID/kidney, 2 hours 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 hours 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 that required euthanasia was reached before day 180. However, mice that had received PMX subther before the injection of $^{99m}$Tc-DMSA 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 Fig. 3A. PMX was applied either in a subtherapeutic dose of 0.4 mg (PMX subther) or at a therapeutic dose of 2 $\times$ 0.8 mg (PMX ther) 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 $\times$ 1 mg per mouse (body weight $\sim$ 25 g) with a time lag of 1 week (Supplementary Methods and Fig. S6; ref. 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 PMX ther (group B: 9.8 $\pm$ 4.1; $P = 0.90$) and in mice treated with $^{177}$Lu-EC0800 and PMX subther (group C: 8.3 $\pm$ 3.5, $P = 0.21$) were not significantly different from the average RTV of control mice (group A: 10.0 $\pm$ 2.7) at day 17 (Table 3). However, the combined application of $^{177}$Lu-EC0800 with PMX ther resulted in a significant decrease of the average RTV (group D: 5.0 $\pm$ 2.2; $P < 0.0001$; Fig. 3B). For tumor model II, the average RTV of PMX-treated mice (group B: 27.9 $\pm$ 11.8; $P = 0.73$) was not significantly reduced compared to control mice (group A: 30.0 $\pm$ 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 $\pm$ 3.9, $P = 0.0006$; group D: 2.0 $\pm$ 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 and E). In contrast, pronounced loss of body weight was observed in group B mice of both tumor models (which received PMX ther 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 an advantage of additive or synergistic antitumor effects and by reducing undesired side effects. This study addressed the question of whether...
PMX contributes to the antitumor 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 folate receptor mediated. It was more pronounced in KB cells, which express the folate receptor at higher levels than IGROV-1 cells, and it was abolished if the cells were coincubated with excess folic acid to block folate receptor binding of $^{177}$Lu-EC0800. Inhibition of cell viability through application of $^{177}$Lu-EC0800 was enhanced if cells were coincubated with PMX (Fig. 1). Determination of the combination indices at different drug concentrations revealed that $^{177}$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 Methods and Fig. S7; refs. 25 and 44). However, exposure of cells to $^{177}$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 $^{177}$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 $^{177}$Lu-EC0800 and PMX.

Figure 3. A, injection scheme of the in vivo therapy studies conducted 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).
enhanced by coapplication of therapeutic amounts of renal accumulation of $^{177}$Lu-EC0800. Analysis of that PMX prevents damage to the kidneys by reducing xenografts. tor–mediated accumulation of $^{177}$Lu-EC0800 in tumor the fact that PMX does not compete with the folate recep-

primarily through carriers such as the reduced folate where-

previously that the tumor uptake of PMX is mediated

understand the underlying mechanism, the uptake of

distribution of $^{177}$Lu-EC0800 dramatically by increasing

compared to the application of each of these therapeu-
tics alone (Supplementary Methods and Fig. S8).

In vivo the preinjection of PMX improved the tissue distribution of $^{177}$Lu-EC0800 dramatically by increasing the tumor-to-kidney ratio (Table 1). In an attempt to understand the underlying mechanism, the uptake of $^3$H-PMX was determined in KB tumors and kidneys of mice. In the kidneys, we found a relatively high uptake 1 hour p.i. of $^3$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 folate receptor (49), support our hypothesis of a competition among PMX and $^{177}$Lu-EC0800 for folate receptor binding sites in the kidneys if PMX was injected about 1 hour before the radiofolate. In the tumor tissue, uptake of $^3$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 and the proton-coupled folate transporter, whereas folate receptors play a minor role (50). This may explain the fact that PMX does not compete with the folate receptor–mediated accumulation of $^{177}$Lu-EC0800 in tumor xenografts.

For the first time we were able to show in this study that PMX prevents damage to the kidneys by reducing renal accumulation of $^{177}$Lu-EC0800. Analysis of plasma parameters and the results of SPECT studies using $^{99m}$Tc-DMSA consistently confirmed normal kidney function in $^{177}$Lu-EC0800-treated mice that 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 $^{177}$Lu-EC0800 on the viability of tumor cells, it is likely that the anticancer effect of $^{177}$Lu-EC0800 would be enhanced by coapplication of therapeutic amounts of PMX ($2 \times 0.8$ mg, corresponding to 80% of the MTD; ref. 16). Therapy studies were conducted with KB (model I) and IGROV-1 tumor-bearing mice (model II) using $^{177}$Lu-EC0800 combined with either subtherapeutic ($0.4$ mg/mouse) or therapeutic doses (e.g., $2 \times 0.8$ mg) of PMX. In both models, tumor growth delay was observed after application of $^{177}$Lu-EC0800. Application of PMX$_{\text{ther}}$ alone showed only minor inhibitory effects on growth of these tumor types. However, PMX$_{\text{ther}}$ was able to enhance the anticancer effect of $^{177}$Lu-EC0800 against both KB and IGROV-1 tumor xenografts. Also, an increased survival time was achieved if $^{177}$Lu-EC0800 and PMX were combined compared to the result obtained with each of these agents applied as monotherapy.

With this study we were able to show the proposed dual effect of PMX in combination with folate receptor targeted radionuclide therapy using $^{177}$Lu-EC0800. On one hand, PMX at subtherapeutic and therapeutic amounts effectively reduced renal uptake of $^{177}$Lu-EC0800 and therewith prevented long-term radionephropathy (Table 1, Fig. 2). On the other hand, the application of PMX$_{\text{ther}}$ alone showed only minor inhibitory effects on growth of these tumor types. However, PMX$_{\text{ther}}$ was able to enhance the anticancer effect of $^{177}$Lu-EC0800 against both KB and IGROV-1 tumor xenografts. Also, an increased survival time was achieved if $^{177}$Lu-EC0800 and PMX were combined compared to the result obtained with each of these agents applied as monotherapy.

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>Group</th>
<th>PMX (mg)</th>
<th>Radioactivity (MBq)</th>
<th>RTV day 17</th>
<th>TGI (%)</th>
<th>TGDI$_4$</th>
<th>Average 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>
</tr>
<tr>
<td>B</td>
<td>$2 \times 0.8$$_{\text{ther}}$</td>
<td>–</td>
<td>9.8 ± 4.1</td>
<td>2.1</td>
<td>1.2</td>
<td>+22.5%</td>
</tr>
<tr>
<td>C</td>
<td>$1 \times 0.4$$_{\text{subther}}$</td>
<td>1 × 20</td>
<td>8.3 ± 3.5</td>
<td>16.9</td>
<td>1.4</td>
<td>+50.0%</td>
</tr>
<tr>
<td>D</td>
<td>$2 \times 0.8$$_{\text{ther}}$</td>
<td>1 × 20</td>
<td>5.0 ± 2.2</td>
<td>50.4</td>
<td>1.9</td>
<td>+75.0%</td>
</tr>
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</table>

Model II: IGROV-1 tumor xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>PMX (mg)</th>
<th>Radioactivity (MBq)</th>
<th>RTV day 17</th>
<th>TGI (%)</th>
<th>TGDI$_4$</th>
<th>Average 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>
</tr>
<tr>
<td>B</td>
<td>$2 \times 0.8$$_{\text{ther}}$</td>
<td>–</td>
<td>27.9 ± 11.8</td>
<td>7.1</td>
<td>1.8</td>
<td>+0%</td>
</tr>
<tr>
<td>C</td>
<td>$1 \times 0.4$$_{\text{subther}}$</td>
<td>1 × 20</td>
<td>7.3 ± 3.9</td>
<td>75.8</td>
<td>2.6</td>
<td>+63.0%</td>
</tr>
<tr>
<td>D</td>
<td>$2 \times 0.8$$_{\text{ther}}$</td>
<td>1 × 20</td>
<td>2.0 ± 1.7</td>
<td>93.2</td>
<td>4.0</td>
<td>+100%</td>
</tr>
</tbody>
</table>

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Reber et al.

Authors’ Contributions
Conception and design: J. Reber, C. Müller
Development of methodology: J. Reber, C. Müller
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Reber, S. Haller, C. Müller
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Reber, C.P. Leamon, C. Müller
Writing, review, and/or revision of the manuscript: J. Reber, S. Haller, C.P. Leamon, C. Müller
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Reber, S. Haller
Study supervision: C. Müller

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


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