Supplementary Methods

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

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Preparation of ^{177}Lu-EC0800. A stock solution of the DOTA-folate conjugate (EC0800, Endocyte Inc., 3 µL, 10^{-3} mol/L) was mixed with HCl (45 µl, 0.05 mol/L), a solution of sodium acetate (10 µL, 0.5 mol/L) and a solution of ascorbic acid (5 µL, 50 mmol/L). No-carrier-added $^{177}LuCl_3$ (Isotope Technologies Garching, ITG GmbH, Munich, Germany) was added in a volume of 3-10 µL (200-300 MBq), and the reaction mixture was incubated for 5 min at 85°C. A solution of Na-DTPA (5 µL, 5 mmol/L, pH 5) was added for complexation of potential traces of unreacted ^{177}Lu(III). Quality control was performed by HPLC using a C-18 reversed phase column (Xterra MS C18, 5 µm, 15 cm x 4.6 cm, Waters). The mobile phase consisted of MilliQ water with 0.1% trifluoroacetic acid (A) and methanol (B) with a linear gradient from 5% A to 80% A in 15 min at a flow rate of 1 mL/min. ^{177}Lu-EC0800 (R_t = 11.7 min) was obtained with a radiochemical yield of >96 % and a specific activity of up to 60 MBq/nmol. The chemical structure of ^{177}Lu-EC0800 is shown in Supplementary Figure S1.

In vitro stability of ^{177}Lu-EC0800. ^{177}Lu-EC0800 (50 µL, 10 MBq) was mixed with phosphate buffered saline (PBS, 950 µL), FFRPMI medium (without supplements) or human blood plasma (250 µL) and incubated at 37°C. Aliquots (50 µL) were taken at different time points (t = 0 h, 4 h and 24 h) after incubation. PBS and FFRPMI samples were directly analyzed using HPLC. For HPLC analysis of blood plasma samples, it was necessary to precipitate proteins which was accomplished by addition of methanol (200 µL) followed by centrifugation.

Cell uptake of ^{177}Lu-EC0800. Cellular uptake and internalization of ^{177}Lu-EC0800 was assessed as previously reported (1). KB, IGROV-1, SKOV-3 and PC-3 cells were seeded in 12-well-plates (700'000 cells per well) the day before the experiments to allow cell adhesion. After removal of the supernantants ^{177}Lu-EC0800 (~35 kBq, 8 pmol) in RPMI or FFRPMI medium (without supplements) was added to each well and the cells were incubated for 4 h at 37°C. Cells were then washed with PBS to determine uptake of ^{177}Lu-EC0800 or with an acidic stripping buffer

(2), for determination of the internalized fraction. Cells were lysed with NaOH (1N) and the cell samples measured for radioactivity in a γ–counter. In order to standardize uptake of radioactivity to the average content of 0.3 mg protein per well the protein content was determined for each sample using a standard protein assay (Micro BCA Protein Assay Kit, Pierce Biotechnology Inc). Supplementary Figure S2 was prepared using *GraphPad Prism* software (version 4).

Clonogenic assay. The clonogenic assay was performed with KB, IGROV-1, SKOV-3 and PC-3 cells according to a previously published procedure (3). A total of 700 cells/well in 2 mL FFRPMI medium (with supplements) were seeded in 6-well plates. After an incubation time of ~15 h allowing cell adhesion, the medium was removed and the cells were incubated with 2 mL FFRPMI medium (without supplements) containing ^{177}Lu-EC0800 (0.001-5.0 MBq/mL). In some cases KB, IGROV-1 and SKOV-3 cells were preincubated with excess folic acid (200 mmol/L) for 1 h to block FRs on the cell surface. Control cells underwent the same procedure but without ^{177}Lu-EC0800. After an incubation time of 4 h at 37°C, the cells were washed once with PBS followed by addition of 2 mL FFRPMI or RPMI medium (with supplements). After two weeks, the medium was removed and cells were washed with 2 mL PBS prior to staining with crystal violet (0.5% crystal violet in 6.0% glutaraldehyde, 2 mL/well). Colonies of a size of at least 0.5 mm were counted manually as previously reported (4) (Supplementary Figure S3). The plating efficiency (PE = number of colonies formed / number of cells seeded x 100%) and the survival fraction (SF = number of colonies formed after treatment / number of cells seeded x PE) were calculated for KB, SKOV-3, IGROV-1 and PC-3 cells.

Calculation of the combination index (CI). The interaction of ^{177}Lu-EC0800 and PMX (Figure 1) was analyzed by determination of the combination index (CI) as it has been recently reported for other combinations by Wouters et al. (5). The calculation of the CI was based on the Chou-Talalay method (6), whereby the CI <1.0 indicates synergism, CI = 1 indicates additivity

and CI >1 indicates antagonism among the two test agents. The CI analysis was performed according to the equation [$CI_x = (D_{comb})_1/(D_{single})_1 + (D_{comb})_2/(D_{single})_2$] where $(D_{single})_1$ and $(D_{single})_2$ are the concentrations of PMX (indexed as 1) and ^{177}Lu-EC0800 (indexed as 2), respectively, that reduce cell viability to x% when applied as single agents and $(D_{comb})_1$ and $(D_{comb})_2$ are the concentrations of PMX (indexed as 1) and ^{177}Lu-EC0800 (indexed as 2), applied in combination that result in the same inhibition of cell viability to x%. The CI_x was calculated for an inhibition of cell viability to x = 55% and x = 70% (Supplementary Table S2).

Biodistribution of ^{177}Lu-EC0800 and dosimetric calculations. For biodistribution studies, athymic nude mice were inoculated with KB cells (5 x 10^6 cells in 100 µl PBS) and IGROV-1 cells (6 x 10^6 cells in 100 µl PBS) into the subcutis of each shoulder. Biodistribution studies were performed approximately 14 days after tumor cell inoculation. ^{177}Lu-EC0800 was formulated in PBS pH 7.4 for immediate administration via a lateral tail vein (3 MBq, 1 nmol, 100 µl per mouse). The solution of PMX (Alimta®) was prepared in NaCl 0.9% according to the instructions of the manufacturer and injected into a lateral tail vein ($PMX_{subther}$: 0.4 mg or PMX_{ther}: 0.8 mg in a volume of 100 µL per mouse), 1 h prior to the injection of ^{177}Lu-EC0800. The animals were sacrificed at different time points after the administration of the radiofolate. Selected tissues and the organs were collected, weighed, and counted for radioactivity in a γ-counter. The results were recorded as the percentage of the injected dose per gram [% ID/g] of tissue weight and the corresponding ratios were calculated.

To estimate the equivalent absorbed radiation dose to the tumor xenografts and kidneys upon injection of ^{177}Lu-EC0800 biodistribution data listed in Supplemental Table S1 (7) were employed to calculate the area under the curve (AUC). For calculation of the renal radiation dose obtained if ^{177}Lu-EC0800 was applied in combination with PMX we estimated a reduction of radioactivity accumulation to ~25% of the value obtained if ^{177}Lu-EC0800 was applied as a single agent. This assumption was based on biodistribution data previously obtained with ^{177}Lu-DOTA-

click-folate conjugate in combination with PMX (8) and on the data obtained with ^{177}Lu-EC0800 in combination with PMX (Table 1). The cumulative radioactivity was calculated from integrated AUCs (MBq·s) of biodistribution data expressed in non-decay-corrected percent injected dose [% ID] per organ mass (100 mg for the tumor, 250 mg for both kidneys). Since biodistribution data were available only over the first 3 days, it was assumed that until day 7 after injection of ^{177}Lu-EC0800, no radioactivity would be left in tumors and kidneys. The adsorbed radiation dose in tumor xenografts was assessed for a sphere of 100 mg using the Unit Density Sphere Model from RADAR (www.doseinfo-radar.com) and the absorbed radiation dose in kidneys was assessed using S-values from Larson et al. for ^{177}Lu (9). The absorbed dose (mGy/MBq) was calculated by multiplying the AUC (s; normalized to 1 MBq ID, Supplementary Figure S4) with the S-value (mGy/MBq·s). Finally the dose (mGy) was calculated by multiplying the equivalent absorbed dose (mGy/MBq) with the amount of injected radioactivity.

Uptake of ^{3}H-PMX in the kidneys and KB tumor xenografts. Investigation of the biodistribution of ^{3}H-PMX was performed in KB tumor-bearing mice. Trace amounts of ^{3}H-PMX (0.5 MBq, <1 µg, 100 µL NaCl 0.9%) were administered into a lateral tail vein. The animals were sacrificed 1 h, 2 h and 4 h p.i. of ^{3}H-PMX. Blood, kidneys and KB tumor xenografts were collected into glass scintillation vials and weighed. After addition of ~3 mL of an aqueous solubilizer (Solvable™, Perkin Elmer) the vials were kept at 50 °C over night allowing digestion of the tissue samples. After cooling the samples to room temperature hydrogen peroxide (300 µL, 30% w/v) was added for decoloration of the solutions. After addition of a scintillation cocktail (15 mL, Ultima Gold™, Perkin Elmer) and homogenization the processed tissue samples were counted for radioactivity in a β-counter. The data were recorded as the percentage of the injected dose per gram [%ID/g] of tissue weight.

Cell cycle analysis. DNA content of KB, IGROV-1 and SKOV-3 cells was measured by flow cytometry to determine the cell cycle distribution upon exposure to ^{177}Lu-EC0800, PMX and the combination of both agents. KB cells (2.5 x 10^5), IGROV-1 (3.5 x 10^5) and SKOV-3 cells (3.5 x 10^5) were seeded in 6-well plates and incubated for 24 h at 37°C allowing cell adhesion. The medium was removed and the cells were incubated in 2 mL FFRPMI medium (without supplements) containing ^{177}Lu-EC0800 and/or PMX for 4 h at 37°C. Afterwards, the medium was removed and the cells were washed with 2 mL PBS before addition of 2 mL FFRPMI medium (with supplements). Cells were then allowed to grow for 24 h at 37°C. Depending on the sensitivity of the tumor cells to ^{177}Lu-EC0800 and PMX, different drug concentrations were applied. KB cells were treated with a low concentration of ^{177}Lu-EC0800 (0.5 MBq/mL) and/or a high dose of PMX (25.0 µM). IGROV-1 cells were treated with a 4-fold higher concentration of ^{177}Lu-EC0800 (2.0 MBq/mL) and/or a lower concentration of PMX (6.00 µM) and SKOV-3 cells were treated with a high concentration of ^{177}Lu-EC0800 (2.0 MBq/mL) and/or a very low concentration of PMX (1.25 µM). Untreated control cells were incubated with FFRPMI medium only. After removal of the medium cells were harvested by trypsinization and fixed with 70% ice-cold ethanol. To determine the DNA content cells were stained with 500 µL of a solution containing 0.05% Triton X-100, RNase A (0.1 mg/mL) and propidium iodine (50 µg/mL) in PBS (pH 7.4). After an incubation time of 40 min at 37°C the cells were washed with PBS pH 7.4 and aliquots of 200 µL were transferred to wells of 96-well plate. Cellular DNA content was analyzed by flow cytometry (Guava EasyCyte Plus Flow Cytometry System, Millipore). At least 10'000 cells were used for each analysis, and the results were analyzed using the software FlowJo (version 7.6.3) (Supplementary Figure S7).

Determination of cell apoptosis. KB, IGROV-1 and SKOV-3 cells (2 x 10^5 cells/well) in 2 mL FFRPMI medium (with supplements) were seeded in 6-well plates. After an incubation time of 24 h at 37°C allowing cell adhesion, the FFRPMI medium was removed. Then, FFRPMI medium

(2 mL, without supplements) containing ^{177}Lu-EC0800 and/or PMX was added and the cells were incubated for 4 h at 37°C. (^{177}Lu-EC0800 and PMX were applied at the same concentrations as employed for determination of the cell cycle distribution in the specific cancer cell). Untreated control cells were incubated with FFRPMI medium (without supplements). After the incubation time of 4 h the medium was removed and the cells were washed with PBS pH 7.4. Cells were then allowed to grow in FFRPMI for 24 h. After removal of the supernatants, cells were trypsinized, washed in PBS (keeping all floating cells) and stained with Guava ViaCount assay (Millipore) according to the manufacturer's instructions. The cell populations were quantified by using a flow cytometer (Guava EasyCyte Plus Flow Cytometry System, Millipore). At least 1'000 cells were used for each measurement, and the results were analyzed using the guavaSoft software (version 2.2) (Supplementary Figure S8).

Determination of the MTD for PMX. Two groups of 4 nude mice each without tumors were intravenously injected with PBS (100 µL, "untreated control mice") or PMX (1 mg/100 µL, "PMX treated mice") at day 0, 7, 14 and 21. Endpoint criteria were defined as body weight loss of over 15% of the initial body weight or abnormal behavior indicating pain or unease. The body weight of each mouse was monitored 3-4 times a week over a time period of 45 days (Supplementary Figure S6) (10).

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