molecular pharmaceutics

articles

Effects of the Antifolates Pemetrexed and CB3717 on the Tissue Distribution of ^{99m}Tc-EC20 in Xenografted and Syngeneic Tumor-Bearing Mice

Cristina Müller,[‡] Joseph A. Reddy,[§] Christopher P. Leamon,[§] and Roger Schibli*[,‡,||]

Center for Radiopharmaceutical Science ETH-PSI-USZ, Paul Scherrer Institute, 5232 Villigen-PSI, Switzerland, Endocyte, Inc., West Lafayette, Indiana 47906, and Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland

Received November 28, 2009; Revised Manuscript Received January 29, 2010; Accepted February 17, 2010

Abstract: Administration of certain antifolates before radiofolate application has previously proven to have a positive effect on undesired kidney uptake of radiofolates in mice bearing human tumor xenografts. The aims of this study were to (i) test the effects of the antifolates, pemetrexed and CB3717, on tissue distribution of the clinically investigated radiofolate, ^{99m}Tc-EC20, and (ii) to determine if pemetrexed's kidney-selective blocking effect also functions in mice bearing syngeneic tumors. Relative binding affinities of pemetrexed and CB3717 were determined in folate receptor (FR)-positive KB cells at 0 and 37 °C using ^{3}H-folic acid. *In vivo* studies were performed in nude mice with KB tumor xenografts (A) and in Balb/c mice bearing FR-positive M109 tumor grafts (B). ^{99m}Tc-EC20 was prepared via a kit formulation. The antifolates pemetrexed and CB3717 (20 μmol/kg body weight) were administered intravenously 1 h before injection of ^{99m}Tc-EC20 (67 nmol/kg body weight). Similar to previously published data we found that FR-binding affinities of pemetrexed and CB3717 at 0 °C were in the same range as that of folic acid. Interestingly, experiments performed at 37 °C showed that pemetrexed has a nearly ~700-fold lower FR-affinity than CB3717. Tissue distribution of ^{99m}Tc-EC20 was largely comparable in both animal models (A and B). Radiofolate accumulation was found in FR-positive tumors (A, 8.92 ± 2.14% ID/g; B, 15.02 ± 0.95% ID/g) and FR-positive kidneys (A, 59.10 ± 8.03% ID/g; B, 69.44 ± 4.66% ID/g, 4 h p.i.). Preinjection of pemetrexed resulted in a significant decrease of ^{99m}Tc-EC20 uptake in kidney (A, 18.80 ± 2.73% ID/g; B, 15.27 ± 2.64% ID/g; 4 h p.i), whereas uptake in the tumors was unaltered. However, administration of the CB3717 resulted in a reduction of ^{99m}Tc-EC20 uptake in both the kidney and tumor (<1% ID/g, 4 h p.i.). We have thus demonstrated that pemetrexed effectively reduces kidney uptake of radiofolates not only in xenografted mice but also in a syngeneic tumor mouse model, thereby indicating that the kidney-specific blocking effect is not based on differences between human and murine FRs that are expressed in xenografts and kidneys, respectively. This effect was not observed with the antifolate, CB3717, which targets the FR selectively in contrast to pemetrexed that is predominantly transported into cells through carrier systems.

Keywords: ^{99m}Tc-EC20; antifolate; pemetrexed; CB3717; folic acid; folate receptor

Introduction

The folate receptor (FR) is expressed in only a limited number of normal tissues (e.g., kidneys, placenta), but frequently overexpressed in cancer, for example, of the ovary, endometrium, breast, colon, stomach, kidneys, and brain.[1,2] The fact that the FR has a high affinity for folic acid (K_D <

* To whom correspondence should be addressed. Mailing address: Paul Scherrer Institute, Center for Radiopharmaceutical Science ETH-PSI-USZ, CH-5232 Villigen-PSI, Switzerland. Email: cristina.mueller@psi.ch. Phone: +41-56-310 44 54. Fax: +41-56-310 28 49.

‡ Paul Scherrer Institute.

§ Endocyte, Inc.

|| ETH Zurich.

(1) Antony, A. C. Folate receptors. *Annu. Rev. Nutr.* **1996**, *16*, 501–521.

10.1021/mp900296k
Published on Web 03/03/2010

1 nM), a property that is retained even in its conjugated state, together with the limited expression in normal tissues, makes it a promising tumor cell surface target to deliver folic acid conjugated diagnostic or therapeutic probes.[3−5]

For potential diagnostic applications in nuclear medicine, several folic acid conjugates suitable for radiolabeling with ^{67}Ga,[6] ^{111}In,[7−9] and ^{99m}Tc[10−16] have been developed for single photon emission computed tomography (SPECT) imaging of malignant tissue. ^{111}In-DTPA-folate was the first radiofolate conjugate tested in patients.[9] However, the development of a ^{99m}Tc-folate conjugate was of more interest because of ^{99m}Tc short half-life and easy availability by a generator system. EC20 is a folate derivative in which an appended short peptide (β-L-diaminopropionate-L-Asp-L-Cys) serves as the site for the tetradentate ^{99m}Tc-chelation. ^{99m}Tc-EC20 showed favorable characteristics in terms of its stability *in vitro* and *in vivo*, high FR-affinity, and most importantly, its promising tissue distribution in M109 tumor-bearing mice.[13,17] Meanwhile ^{99m}Tc-EC20 is being clinically tested in several countries for imaging of FR-positive tumors in patients that could potentially benefit from FR-targeted chemotherapies.[18]

Although FR expression is limited in normal tissues, its expression in the proximal tubule cells of the kidneys, which serves for reabsorption of physiological folates,[19,20] may be a problematic issue for the FR-targeting of some agents. Renal FRs are targeted concomitantly to tumor-associated FRs by small folic acid conjugates that are filtered via the glomeruli. Accumulation of radioactivity (percent injected dose per gram tissue [%ID/g]) in the kidneys is usually around 10-fold higher than accumulation in FR-positive tumor (xeno)grafts. Thus, tumor-to-kidney ratios of radioactivity are generally low for all reported radiofolates. For imaging purposes, retention of radioactivity in the kidneys is not a handicap for a clinical application. However, in view of a development of folate-based radionuclide therapy, high activity retention in the renal tissue is highly undesirable due to the risk of radiation induced nephrotoxicity.

Previously, we have reported that preinjection of certain antifolates (pemetrexed, raltitrexed, methotrexate) can selectively reduce radiofolate uptake in the kidneys while the uptake in FR-positive KB tumor xenografts was not affected resulting in significantly improved tumor-to-kidney ratios of radioactivity.[21−23] Interestingly, this effect was most favor-

(2) Garin-Chesa, P.; Campbell, I.; Saigo, P. E.; Lewis, J. L.; Old, L. J.; Rettig, W. J. Trophoblast and ovarian cancer antigen LK26 - sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am. J. Pathol.* **1993**, *142* (2), 557–567.

(3) Low, P. S.; Antony, A. C. Folate receptor-targeted drugs for cancer and inflammatory diseases - Preface. *Adv. Drug Delivery Rev.* **2004**, *56* (8), 1055–1058.

(4) Hilgenbrink, A. R.; Low, P. S. Folate receptor-mediated drug targeting: From therapeutics to diagnostics. *J. Pharm. Sci.* **2005**, *94* (10), 2135–2146.

(5) Salazar, M. D.; Ratnam, M. The folate receptor: What does it promise in tissue-targeted therapeutics. *Cancer Metastasis Rev.* **2007**, *26* (1), 141–152.

(6) Mathias, C. J.; Wang, S.; Low, P. S.; Waters, D. J.; Green, M. A. Receptor-mediated targeting of ^{67}Ga-deferoxamine-folate to folate-receptor-positive human KB tumor xenografts. *Nucl. Med. Biol.* **1999**, *26* (1), 23–25.

(7) Wang, S.; Luo, J.; Lantrip, D. A.; Waters, D. J.; Mathias, C. J.; Green, M. A.; Fuchs, P. L.; Low, P. S. Design and synthesis of [^{111}In]DTPA-folate for use as a tumor-targeted radiopharmaceutical. *Bioconjugate Chem.* **1997**, *8* (5), 673–679.

(8) Mathias, C. J.; Wang, S.; Waters, D. J.; Turek, J. J.; Low, P. S.; Green, M. A. Indium-111-DTPA-folate as a potential folate-receptor-targeted radiopharmaceutical. *J. Nucl. Med.* **1998**, *39* (9), 1579–1585.

(9) Siegel, B. A.; Dehdashti, F.; Mutch, D. G.; Podoloff, D. A.; Wendt, R.; Sutton, G. P.; Burt, R. W.; Ellis, P. R.; Mathias, C. J.; Green, M. A.; Gershenson, D. M. Evaluation of ^{111}In-DTPA-folate as a receptor-targeted diagnostic agent for ovarian cancer: initial clinical results. *J. Nucl. Med.* **2003**, *44* (5), 700–707.

(10) Guo, W. J.; Hinkle, G. H.; Lee, R. J. ^{99m}Tc-HYNIC-folate: a novel receptor-based targeted radiopharmaceutical for tumor imaging. *J. Nucl. Med.* **1999**, *40* (9), 1563–1569.

(11) Mathias, C. J.; Hubers, D.; Low, P. S.; Green, M. A. Synthesis of [^{99m}Tc]DTPA-folate and its evaluation as a folate-receptor-targeted radiopharmaceutical. *Bioconjugate Chem.* **2000**, *11* (2), 253–257.

(12) Trump, D. P.; Mathias, C. J.; Yang, Z. F.; Low, P. S. W.; Marmion, M.; Green, M. A. Synthesis and evaluation of ^{99m}Tc(CO)$_3$-DTPA-folate as a folate-receptor-targeted radiopharmaceutical. *Nucl. Med. Biol* **2002**, *29* (5), 569–573.

(13) Leamon, C. P.; Parker, M. A.; Vlahov, I. R.; Xu, L. C.; Reddy, J. A.; Vetzel, M.; Douglas, N. Synthesis and biological evaluation of EC20: a new folate- derived, ^{99m}Tc-based radiopharmaceutical. *Bioconjugate Chem.* **2002**, *13* (6), 1200–1210.

(14) Müller, C.; Hohn, A.; Schubiger, P. A.; Schibli, R. Preclinical evaluation of novel organometallic ^{99m}Tc-folate and ^{99m}Tc-pteroate radiotracers for folate receptor-positive tumour targeting. *Eur. J. Nucl. Med. Mol. Imaging* **2006**, *33* (9), 1007–1016.

(15) Müller, C.; Schubiger, P. A.; Schibli, R. Synthesis and in vitro/in vivo evaluation of novel ^{99m}Tc(CO)$_3$-folates. *Bioconjugate Chem.* **2006**, *17* (3), 797–806.

(16) Mindt, T. L.; Müller, C.; Melis, M.; de Jong, M.; Schibli, R. Click-to-chelate": in vitro and in vivo comparison of a ^{99m}Tc(CO)$_3$-labeled N(τ)-histidine folate derivative with its isostructural, clicked 1,2,3-triazole analogue. *Bioconjugate Chem.* **2008**, *19* (8), 1689–95.

(17) Reddy, J. A.; Xu, L. C.; Parker, N.; Vetzel, M.; Leamon, C. P. Preclinical evaluation of ^{99m}Tc-EC20 for imaging folate receptor-positive tumors. *J. Nucl. Med.* **2004**, *45* (5), 857–866.

(18) Fisher, R. E.; Siegel, B. A.; Edell, S. L.; Oyesiku, N. M.; Morgenstern, D. E.; Messmann, R. A.; Amato, R. J. Exploratory study of ^{99m}Tc-EC20 imaging for identifying patients with folate receptor-positive solid tumors. *J. Nucl. Med.* **2008**, *49* (6), 899–906.

(19) Goresky, C. A.; Watanabe, H.; Johns, D. G. The renal excretion of folic acid. *J. Clin. Invest.* **1963**, *42*, 1841–9.

(20) Holm, J.; Hansen, S. I.; Hoiermadsen, M.; Bostad, L. A high-affinity folate binding-protein in proximal tubule cells of human kidney. *Kidney Int.* **1992**, *41* (1), 50–55.

(21) Müller, C.; Brühlmeier, M.; Schubiger, A. P.; Schibli, R. Effects of antifolate drugs on the cellular uptake of radiofolates in vitro and in vivo. *J. Nucl. Med.* **2006**, *47* (12), 2057–2064.

(22) Müller, C.; Schubiger, P. A.; Schibli, R. Isostructural folate conjugates radiolabeled with the matched pair ^{99m}Tc/^{188}Re: a potential strategy for diagnosis and therapy of folate receptor-positive tumors. *Nucl. Med. Biol.* **2007**, *34* (6), 595–601.

ably achieved with pemetrexed (PMX) whereas the application of methotrexate did not lead to a likewise beneficial outcome. Administration of PMX also had a beneficial impact on the tissue distribution of radiofolates in nude mice with subcutaneous solid human ovarian tumor xenografts (IGROV-1) and in an ovarian cancer metastases model of intraperitoneally injected human SKOV-3 cells.[24] PMX is an antifolate of the newer generation that targets multiple folate-dependent enzymes, including thymidylate synthase. PMX has been commercialized as an anticancer drug for the treatment of malignant mesothelioma and nonsmall cell lung cancer, and it is currently in clinical trials for the treatment of several other cancer types.[25] While the chemical structure of PMX (as well as ralitrexed and methotrexate) is based on the structure of folates, the kidney-specific results obtained with PMX in combination with radiofolates could not be achieved by a preinjection of folic acid or its reduced counterpart leucovorin.[26] The uptake of PMX and also of raltiterxed and methotrexate into (malignant) cells is predominantly mediated by the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT), whereas the FR plays a secondary role in this respect. Nevertheless, the literature reports a high FR-affinity for PMX similar to that of folic acid, which is in contrast to methotrexate that is known to bind to the FR with at least 100-fold lower affinity.[27−29]

In this study, we wanted to investigate whether the effect of a selective reduction of radioactivity accumulation in the kidneys could be likewise achieved by an antifolate with different cell uptake characteristics as compared to PMX, methotrexate, and raltiterxed. The compound CB3717 was one of the first FR-selective antifolates that featured a relatively low affinity for the RFC.[30] Therefore, we compared the effect of PMX with that of CB3717 with the aim to investigate which feature of a particular antifolate makes it suitable for application in combination with radiofolates.

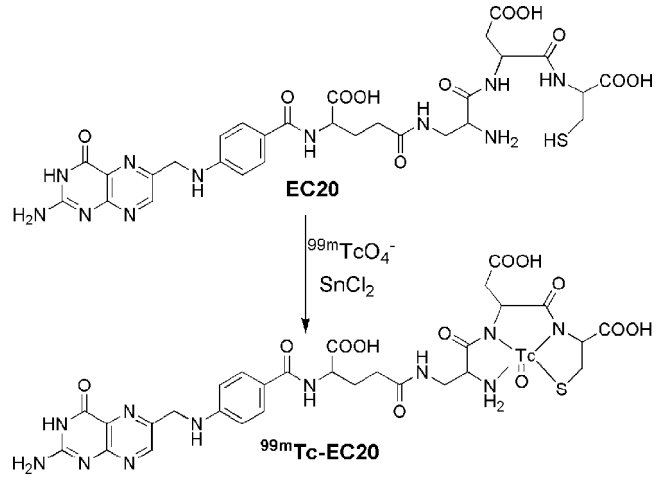

Figure 1. Chemical structure of EC20 and preparation of ^{99m}Tc-EC20.

Second, we proposed to answer the question whether the effect of a selective kidney blockade after the injection of PMX was caused by potentially different affinities of PMX to the human FR expressed in human tumor xenografts (e.g., KB tumors) and the mouse FR expressed in the kidneys of the test animals. Therefore, we tested the effect of PMX on radiofolate tissue distribution in a syngeneic mouse model bearing tumor grafts that express the murine FR. Importantly, for all of the *in vivo* studies described herein, the clinically investigated radiofolate, ^{99m}Tc-EC20, was used.

Experimental Section

Radiolabeling of EC20. Preformulated kits were used for the preparation of the ^{99m}Tc-EC20 (Figure 1). Each kit contained a sterile, nonpyrogenic lyophilized mixture of 0.1 mg of EC20, and additives such as sodium a-D-glucoheptonate, tin(II)chloride dihydrate, and sufficient sodium hydroxide or hydrochloric acid to adjust the pH to 6.8 ± 0.2 before lyophilization. ^{99m}Tc-EC20 was prepared by addition of [Na][$^{99m}TcO_4$] (1−2 GBq) to the EC20 kit vial in a volume of 2 mL. The vial was then incubated at 100 °C for 18 min. Quality control was performed by HPLC (Merck-Hitachi L-6200A system equipped with an L-3000 tunable absorption detector, a Berthold LB 508 radiometric detector) and an XTerra MS C18 reversed-phase column (5 μm, 15 cm × 4.6 mm; Waters). The eluents consisted of aqueous 0.05 M triethylammonium phosphate buffer, pH 2.25, and methanol with a linear gradient from 5 to 80% methanol over 15 min. For the *in vivo* application, the ^{99m}Tc-EC20 solution was further diluted with 0.9% NaCl.

Cell Culture. KB cells (human nasopharyngeal carcinoma cell line) were purchased from American Type Culture

(23) Müller, C.; Mindt, T. L.; de Jong, M.; Schibli, R. Evaluation of a novel radiofolate in tumour-bearing mice: promising prospects for folate-based radionuclide therapy. *Eur. J. Nucl. Med. Mol. Imaging* **2009**, *36* (6), 938–46.

(24) Müller, C.; Schibli, R.; Krenning, E. P.; de Jong, M. Pemetrexed improves tumor selectivity of ^{111}In-DTPA-folate in mice with folate receptor-positive ovarian cancer. *J. Nucl. Med.* **2008**, *49* (4), 623–9.

(25) Paz-Ares, L.; Bezares, S.; Tabernero, J. M.; Castellanos, D.; Cortes-Funes, H. Review of a promising new agent - pemetrexed disodium. *Cancer* **2003**, *97* (8), 2056–2063.

(26) Müller, C.; Schibli, R.; Forrer, F.; Krenning, E. P.; de Jong, M. Dose-dependent effects of (anti)folate preinjection on ^{99m}Tc-radiofolate uptake in tumors and kidneys. *Nucl. Med. Biol.* **2007**, *34* (6), 603–8.

(27) Westerhof, G. R.; Schornagel, J. H.; Kathmann, I.; Jackman, A. L.; Rosowsky, A.; Forsch, R. A.; Hynes, J. B.; Boyle, F. T.; Peters, G. J.; Pinedo, H. M.; Jansen, G. Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular structure and biological activity. *Mol. Pharmacol.* **1995**, *48* (3), 459–471.

(28) Theti, D. S.; Jackman, A. L. The role of α-folate receptor-mediated transport in the antitumor activity of antifolate drugs. *Clin. Cancer Res.* **2004**, *10* (3), 1080–1089.

(29) Jackman, A. L.; Theti, D. S.; Gibbs, D. D. Antifolates targeted specifically to the folate receptor. *Adv. Drug Delivery Rev.* **2004**, *56* (8), 1111–1125.

(30) Jackman, A. L.; Calvert, A. H. Folate-based thymidylate synthase inhibitors as anticancer drugs. *Ann. Oncol.* **1995**, *6* (9), 871–81.

Collection (CCL-17; ATCC, Manassas, U.S.) and M109 cells were a gift from Dr. Alberto Gabizon (Shaare Zedek Medical Center, Jerusalem, Israel). The cells were cultured as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2. Importantly, the cells were cultured in a folate-free cell culture medium, FFRPMI (modified RPMI, without folic acid, vitamin B_{12}, and phenol red; Cell Culture Technologies GmbH, Gravesano/Lugano, Switzerland). FFRPMI medium was supplemented with 10% heat-inactivated fetal calf serum (FCS, as the only source of folate), L-glutamine, and antibiotics (penicillin/streptomycin/fungizone). Routine culture treatment was performed twice a week.

Relative Affinity at 0 and 37 °C. The relative affinities of PMX and CB3717 were determined according to the method described by Westerhoff et al.[27] with slight modification. Briefly, FR-positive KB cells were seeded in 24-well Falcon plates and allowed to form adherent monolayers (>75% confluent) overnight in FFRPMI/FCS. Spent incubation media was replaced with FFRPMI supplemented with 10% FCS and containing 100 nM of ^{3}H-folic acid in the absence and presence of increasing concentrations of unlabeled folic acid, PMX, or CB3717. Cells were incubated for 1 h at 0 and 37 °C, respectively, and then rinsed 3 times with 0.5 mL of PBS. Five hundred microliters of 1% sodium dodecylsulfate in PBS was added to each well. After 5 min, cell lysates were collected, transferred to individual vials containing 5 mL of scintillation cocktail, and then counted for radioactivity. Cells exposed to only the ^{3}H-folic acid in FFRPMI (no competitor) were designated as negative controls, whereas cells exposed to the ^{3}H-folic acid plus 1 mM unlabeled folic acid served as positive controls. Disintegrations per minute (DPMs) measured in the latter samples (representing nonspecific binding of label) were subtracted from the DPM values from all samples. Notably, relative affinities were defined as the inverse molar ratio of compound required to displace 50% of ^{3}H-folic acid bound to FR on KB cells, and the relative affinity of folic acid for the FR was set to 1.

Biodistribution Studies. All animal experiments were approved by the governing Animal Welfare Committee and conducted in accordance with the regulations of the Paul Scherrer Institute, Switzerland and American Accreditation Association of Laboratory Animal Care guidelines. Four to five-week-old female, athymic nude mice (NMRI *nu/nu*) were purchased from Charles River Laboratories (Sulzfeld, Germany). BALB/c mice were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, Indiana). The animals were fed with a folate-deficient rodent diet starting 5−7 days prior to the tumor cell inoculation.[31] Mice were inoculated with FR-positive KB cells (5×10^6 cells) or syngeneic FR-positive M109 tumor cells (1×10^6 cells) in the subcutis of the axilla. Animal experiments were performed 2 or 3 weeks after tumor cell inoculation, when the tumor reached a size of approximately 0.5−1 cm^3.

All biodistribution studies were performed in triplicate. The animals received an intravenous dose of ^{99m}Tc-EC20 (45 nmol/kg or 67 nmol/kg, as indicated) in a volume of 100 μL via a lateral tail vein. PMX (pemetrexed; Alimta, LY231514; Eli Lilly, Bad Homburg, Germany) was diluted with 0.9% NaCl according to the instruction of the manufacturer. The antifolate CB3717 (provided by Endocyte Inc.) was diluted in PBS pH 7.4. The antifolates were administered into a lateral tail vein 1 h prior to ^{99m}Tc-EC20. The animals were sacrificed at indicated points in time after administration of the radiofolate. Selected tissues and organs were removed and weighed, and their radioactivity content was measured in a γ-counter to determine ^{99m}Tc distribution. The results were calculated as percentage of the injected dose per gram of tissue weight [% ID/g] by reference to standards prepared from dilutions of the injected preparation counted at the same time. Statistical analyses were 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 statistically significant.

Results

Radiolabeling. ^{99m}Tc-labeling of EC20 was performed by addition of [Na][$^{99m}TcO_4$] to the kit formulation (Figure 1). Incorporation of the radionuclide was practically quantitative within 20 min. Thus, radiochemical purity of ^{99m}Tc-EC20 was commonly >98%. A purification step before application was therefore not necessary. Molar doses refer to the total injected amount (i.e., ^{99m}Tc-EC20 plus unlabeled EC20).

Relative Affinity of PMX and CB3717 at 0 and 37 °C. Newly developed folate conjugates are usually analyzed by an *in vitro* relative affinity assay that measures a ligand's ability to directly compete with folic acid for binding to cell surface exposed FRs. In this study, the relative affinities of PMX and CB3717 were tested in comparison to folic acid at 0 and 37 °C (Figure 2).

Previously, it was shown by Westerhof et al. that CB3717 and PMX bind with high affinity to the FR.[27] Our results of the experiments performed at 37 °C showed that the relative affinity of CB3717 (1.01) was in the same range as that of folic acid (1.00), whereas PMX (0.0015) showed a nearly 700-fold lower relative affinity. When the relative affinity of PMX was tested at 0 °C, our results were similar to those of Westerhof et al. (Figure 3).

Biodistribution Studies. Biodistribution studies were performed in KB tumor-bearing nude mice at different time points (1, 4, and 24 h) after injection of ^{99m}Tc-EC20 (45 nmol/kg) with and without preinjection of PMX (Table 1). Similar to our previous studies, PMX was administered at a dose of approximately 34 μmol/kg body weight. Radioactive uptake in the tumor was saturated within 1 h after injection (11.39 ± 3.24% ID/g) and was retained over the first few hours. Even on the following day (24 h p.i.), radiofolate retention in the tumor was still prominent (7.02 ± 1.10%

(31) Mathias, C. J.; Wang, S.; Lee, R. J.; Waters, D. J.; Low, P. S.; Green, M. A. Tumor-selective radiopharmaceutical targeting via receptor-mediated endocytosis of gallium-67-deferoxamine-folate. *J. Nucl. Med.* **1996**, *37* (6), 1003–1008.

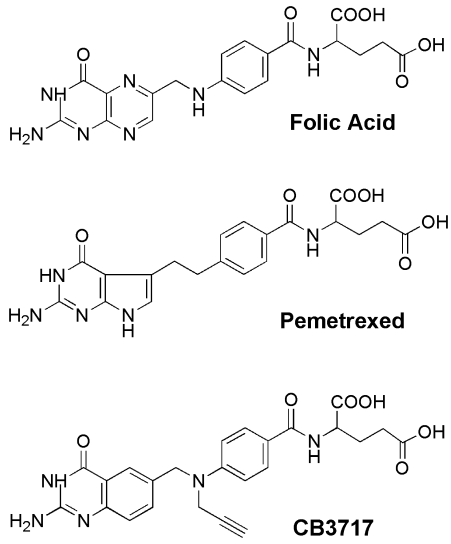

Figure 2. Chemical structures of folic acid, pemetrexed, and CB3717.

ID/g). Tumor uptake in mice predosed with PMX was comparable to that in the control group that did not receive PMX. Radiofolate uptake in the kidneys was extremely high in the control group (~90% ID/g, 4 h p.i.) as a result of radiofolate binding to FRs that are expressed on the proximal tubule cells. However, a tremendous reduction of ^{99m}Tc-EC20 uptake was observed in the kidneys (9.44 ± 0.39% ID/g; 4 h p.i.) of mice that had been preinjected with PMX.

In the next experiment, an ~50% higher molar amount of ^{99m}Tc-EC20 (67 nmol/kg) was administered in combination with variable molar amounts of preinjected PMX. None of the three PMX doses tested (8.5, 17, and 34 μmol/kg body weight) were found to have any impact on the tumor uptake, but kidney retention was significantly reduced with all three doses of PMX, with the greatest reduction in the highest PMX (34 μmol/kg) dose group. Tumor-to-kidney ratios of radioactivity were significantly increased in all the groups predosed with PMX as compared to control groups, while different doses of PMX altered this ratio insignificantly (Figure 4).

Tissue distribution studies were performed 4 h after injection of ^{99m}Tc-EC20 (67 nmol/kg body weight) in nude mice bearing KB-tumor xenografts (**A**) that express the human FR, and Balb/c mice with syngeneic M109 tumor grafts that express an endogenous murine FR (**B**) (Table 2). In addition to ^{99m}Tc-EC20, three mice from each of the tumor mouse models received a preinjection of PMX or CB3717 at the same molar amount (20 μmol/kg body weight). Data of control mice were largely the same for both KB and M109 tumor-bearing mice. Radioactivity accumulation was basically found in FR-positive tumors (**A**, 8.92 ± 2.14% ID/g; **B**, 15.02 ± 0.95% ID/g) and kidneys (**A**, 59.10 ± 8.03% ID/g; **B**, 69.44 ± 4.66% ID/g). Importantly, preinjection of PMX had significantly reduced kidney uptake in both animal models (**A**, 18.80 ± 2.73% ID/g; **B**, 15.27 ± 2.64% ID/g), whereas the tumor uptake was unaltered; thus, the tumor-to-kidney ratios increased approximately 3-fold (**A**, 0.57 ± 0.07; **B**, 0.70 ± 0.19). For both tumor mouse models, the tumor-to-blood and tumor-to-liver ratios were in the same range as that found in control mice. Interestingly, the effect achieved by PMX could not be reproduced in mice that received preinjected CB3717 in either of the animal models. Although kidney uptake was reduced to 10% of control values after the injection of CB3717, the tumor uptake also decreased concomitantly, resulting in values of less than 1% ID/g. Consequently, tumor-to-background ratios were even smaller than those in control mice.

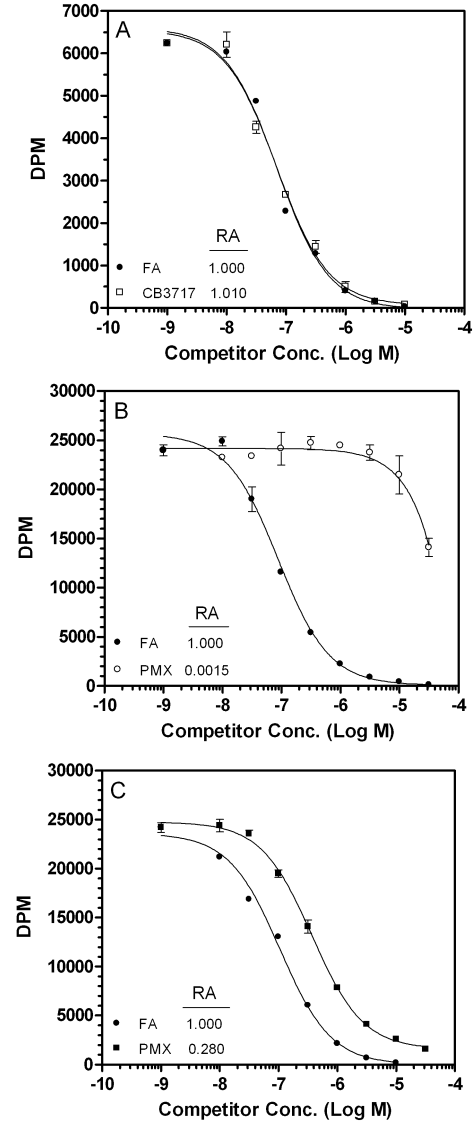

Figure 3. Relative affinity (RA) of CB3717 and pemetrexed to FRs on KB cells compared to folic acid at 37 °C (panel A and B) and at 0 °C (panel C).

Discussion

In various studies performed with different radiofolates, we were able to show that preinjected PMX reduced renal uptake of radioactivity while the tumor uptake was not affected.[21,23,24] In this study, we used a clinically tested radiofolate (^{99m}Tc-EC20) to compare the effects of PMX and the antifolate CB3717 on its tissue distribution. PMX is an

Table 1. Biodistribution of ^{99m}Tc-EC20 (45 nmol/kg) with/without Preinjection of PMX (34 μmol/kg) in KB-Tumor Bearing Female Nude Mice

	1 h p.i.	4 h p.i.	24 h p.i.
blood	0.38 ± 0.12	0.20 ± 0.01	0.08 ± 0.01
lung	1.14 ± 0.24	0.81 ± 0.22	0.31 ± 0.02
spleen	0.46 ± 0.12	0.27 ± 0.06	0.08 ± 0.01
kidneys[b]	**89.18 ± 10.73**	**90.57 ± 11.88**	**28.26 ± 5.70**
stomach	1.60 ± 0.32	0.97 ± 0.23	0.43 ± 0.06
intestines	0.95 ± 0.38	0.51 ± 0.04	0.18 ± 0.05
liver	5.12 ± 1.57	3.06 ± 0.48	0.59 ± 0.10
muscle	0.73 ± 0.10	0.49 ± 0.15	0.19 ± 0.05
bone	0.70 ± 0.18	0.35 ± 0.03	0.16 ± 0.02
salivary glands	8.08 ± 2.51	2.80 ± 0.80	0.61 ± 0.03
tumor	**11.39 ± 3.24**	**12.26 ± 1.05**	**7.02 ± 1.10**
tumor-to-blood	30.09 ± 3.39	59.56 ± 4.22	89.57 ± 8.95
tumor-to-liver	2.28 ± 0.58	3.40 ± 1.35	11.96 ± 1.36
tumor-to-kidney	**0.13 ± 0.04**	**0.14 ± 0.03**	**0.26 ± 0.09**

	pemetrexed[a]		
	1 h p.i.	4 h p.i.	24 h p.i.
blood	0.40 ± 0.12	0.14 ± 0.02	0.07 ± 0.02
lung	1.00 ± 0.29	0.65 ± 0.04	0.24 ± 0.03
spleen	0.40 ± 0.11	0.18 ± 0.01	0.07 ± 0.02
kidneys	**19.14 ± 5.16**	**9.44 ± 0.39**	**4.58 ± 0.37**
stomach	1.32 ± 0.24	0.73 ± 0.05	0.28 ± 0.02
intestines	0.82 ± 0.18	0.48 ± 0.12	0.11 ± 0.04
liver	4.66 ± 0.77	2.18 ± 0.31	0.37 ± 0.04
muscle	0.61 ± 0.07	0.30 ± 0.03	0.10 ± 0.02
bone	0.78 ± 0.24	0.29 ± 0.05	0.10 ± 0.01
salivary glands	3.88 ± 0.74	1.56 ± 0.31	0.36 ± 0.04
tumor	**12.16 ± 2.40**	**11.47 ± 1.38**	**5.66 ± 0.80**
tumor-to-blood	28.84 ± 2.29	81.66 ± 16.03	82.22 ± 6.63
tumor-to-liver	2.60 ± 0.21	5.37 ± 1.18	15.04 ± 1.08
tumor-to-kidney	**0.65 ± 0.10**	**1.22 ± 0.17**	**1.27 ± 0.30**

[a] Injected 1 h prior to ^{99m}Tc-EC20. [b] Values of particular interest are marked in bold.

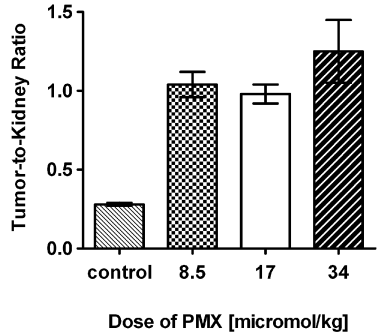

Figure 4. Tumor-to-kidney ratios of radioactivity after injection of pemetrexed at variable doses between 8.5 and 34 μmol/kg body weight.

antifolate that targets multiple enzymes involved in both the pyrimidine and purine syntheses. Clinical trials with PMX were encouraging, and antitumor activity was found in a wide range of cancer types, including nonsmall cell lung cancer, malignant mesothelioma, and carcinoma of the breast, colorectum, uterine cervix, head, and neck as well as of the bladder.[25] On the other hand, CB3717 is an antifolate that was clinically evaluated as the first folate-based thymidylate synthase inhibitor.[30] Despite its promising antitumor activity, CB3717 was not developed further due to sporadic toxicity.

Table 2. Biodistribution of ^{99m}Tc-EC20 (67 nmol/kg) in KB-tumor (**A**) and M109 tumor bearing mice (**B**) with/without preinjection of PMX (20 μmol/kg) and CB3717 (20 μmol/kg), respectively

	KB tumor model (**A**)		
	control	pemetrexed[a]	CB3717[a]
	4 h p.i.	4 h p.i.	4 h p.i.
blood	0.15 ± 0.03	0.21 ± 0.07	0.09 ± 0.04
heart	0.82 ± 0.09	0.56 ± 0.05	0.06 ± 0.02
lung	0.63 ± 0.04	0.59 ± 0.10	0.14 ± 0.04
spleen	0.26 ± 0.02	0.24 ± 0.02	0.12 ± 0.02
kidneys[b]	**59.10 ± 8.03**	**18.80 ± 2.73**	**5.74 ± 1.01**
stomach	0.70 ± 0.05	0.68 ± 0.07	0.15 ± 0.04
intestines	0.47 ± 0.06	0.44 ± 0.09	0.28 ± 0.04
liver	2.27 ± 0.13	2.04 ± 0.12	0.91 ± 0.20
muscle	0.47 ± 0.16	0.34 ± 0.07	0.07 ± 0.08
tumor	**8.92 ± 2.14**	**10.17 ± 2.27**	**0.30 ± 0.03**
tumor-to-blood	61.24 ± 21.63	47.35 ± 7.81	3.50 ± 0.96
tumor-to-liver	3.96 ± 1.13	4.27 ± 2.57	0.33 ± 0.04
tumor-to-kidney	**0.15 ± 0.05**	**0.57 ± 0.07**	**0.05 ± 0.00**

	M109 tumor model (**B**)		
	control	pemetrexed[a]	CB3717[a]
	4 h p.i.	4 h p.i.	4 h p.i.
blood	0.20 ± 0.01	0.20 ± 0.02	0.16 ± 0.05
heart	1.39 ± 0.10	1.34 ± 0.19	0.15 ± 0.06
lung	1.62 ± 0.09	1.59 ± 0.16	0.41 ± 0.19
spleen	0.34 ± 0.05	0.41 ± 0.21	0.24 ± 0.08
kidneys	**69.44 ± 4.66**	**15.27 ± 2.64**	**8.69 ± 6.65**
stomach	0.91 ± 0.17	0.80 ± 0.28	0.21 ± 0.15
intestines	2.64 ± 0.36	3.22 ± 0.22	4.28 ± 1.66
liver	4.58 ± 0.48	3.82 ± 0.28	2.03 ± 0.74
muscle	1.59 ± 0.18	1.48 ± 0.55	0.19 ± 0.09
tumor	**15.02 ± 0.95**	**10.49 ± 2.41**	**0.79 ± 0.42**
tumor-to-blood	76.23 ± 7.26	53.16 ± 13.72	4.72 ± 1.51
tumor-to-liver	3.30 ± 0.36	2.78 ± 0.81	0.38 ± 0.07
tumor-to-kidney	**0.22 ± 0.03**	**0.70 ± 0.19**	**0.10 ± 0.02**

[a] Injected 1 h prior to ^{99m}Tc-EC20. [b] Values of particular interest are marked in bold.

^{99m}Tc-EC20 is a folate-based radiotracer that has advanced to clinical phase II studies for the determination of FR-positive cancer in patients. In the study reported herein, the PMX "effect" observed in combination with other radiofolates in previous studies was also reproduced with ^{99m}Tc-EC20. Thus, preinjection of PMX resulted in a significant reduction of ^{99m}Tc-EC20 kidney uptake without compromising the tumor accumulation to significantly improve the tumor-to-kidney ratio. A 50% increase in ^{99m}Tc-EC20 dose (45 vs 67 nmol/kg body weight) had some impact on the radioactivity uptake in the kidneys (90.57 ± 11.88 vs 59.10 ± 8.03% ID/g; 4 h p.i.), while the tumor uptake remained the same. These findings were in line with those from Mathias et al. who found that variable molar amounts of

injected ^{67}Ga-deferoxamine-folate resulted in a different uptake of radioactivity in the kidneys.[6] A tremendous reduction of kidney uptake (to 10% of control values) was found in mice that received PMX (34 μmol/kg body weight) one hour before ^{99m}Tc-EC20 (45 nmol/kg body weight). This effect was less pronounced in mice that received a lower molar dose of PMX (20 μmol/kg body weight) or a higher molar amount of ^{99m}Tc-EC20 (67 nmol/kg body weight). In the latter study, kidney uptake was reduced to only 30% of control values indicating that the molar amount of PMX relative to that of the radiofolate plays an important role in the level of ^{99m}Tc-EC20 reduction in the renal uptake. This in turn might indicate a competition among the two substances, the antifolate and the radiofolate, respectively, for FR-binding sites in the proximal tubule cells of the kidneys. However, the tumor uptake of ^{99m}Tc-EC20 was not affected at all by preinjected PMX, and a significant difference could not be observed among the two experiments employing variable molar amounts of PMX and radiofolate, respectively. As a result an increased tumor-to-kidney ratio was found for all experiments performed with PMX with values that varied between 0.57 ± 0.07 and 1.22 ± 0.17. The fact that the tumor-to-blood and tumor-to-liver ratios of the group of mice that received PMX remained in the same range as those from animals of the control group suggests that the attenuation of radiofolate uptake after PMX injection was limited to the renal tissue.

Interestingly, the studies presented herein that were performed in combination with CB3717 revealed completely different results than those obtained with PMX. After injection of CB3717, the uptake of ^{99m}Tc-EC20 was even more reduced in the kidneys than after injection of PMX. Undesirably, the tumor uptake of the radiofolate was concomitantly blocked. Radioactivity retention in the tumor was less than 1% ID/g, 4 h after injection of ^{99m}Tc-EC20. The overall tissue distribution pattern of ^{99m}Tc-EC20 after injection of CB3717 resembled much more the tissue distribution pattern after injection of an excess folic acid than that after PMX. According to the literature, FR-binding affinity of CB3717 is in the same range as folic acid and its affinity to the reduced folate carrier (RFC) is only poor making CB3717 (similar to folic acid), a more selective compound for the FR than PMX.[27] Also, FR-binding of PMX was reported to be high, similar to that of CB3717 and folic acid.[27] Our findings of the FR-binding affinity experiments performed at 0 °C were comparable to literature values. However, when we tested FR-binding affinity of these antifolates at a physiological temperature of 37 °C, we found that the relative affinity of CB3717 was still comparable to that of folic acid, whereas that of PMX was more than two magnitudes lower. Since under physiological conditions pharmacological effects such as carrier-mediated (anti)folate transport mechanisms and intracellular processes such as enzyme reactions are active, it is most likely that differences in affinities among PMX and CB3717 at 37 °C are related to their dissimilar FR-selectivity. Theti et al. reported that cellular uptake of PMX (and other antifolates such as methotrexate) was mediated basically via carrier systems.[28] Principally, these are the RFC and the more recently identified proton-coupled folate transporter (PCFT).[32] Our observations of different FR-binding affinities of PMX at 0 and 37 °C, respectively, could be the result of an interference of carrier systems in the uptake of PMX at 37 °C. Also, it was proposed by Mauritz et al. that polyglutamylation might be a driving force to release antifolates from receptors after FR-mediated cell membrane translocation.[33] Since PMX is known to be an excellent substrate for folylpolyglutamate synthetase, its polyglutamylation might add to the fact that under physiological conditions the concentration of PMX decreases rapidly. The consequence would be that an increased amount of PMX was necessary to block FRs leading to the result of an apparently lower relative FR-affinity. Thus, the differences among PMX and CB3717 in binding affinities to folate transport systems, other than the FR, and to intracellular enzymes might be an explanation for their different behavior in combination with radiofolates *in vivo*.

In our previous studies with radiofolates in combination with antifolates we had used human tumor xenografts. Mice with xenografts of KB tumor cells, a human nasopharyngeal carcinoma cell line, are routinely used as a standard animal tumor model for FR-targeting research because of the high FR-expression level of these tumor cells. Ovarian cancer is the primary target of FR-targeted therapy methods since FR-overexpression has been observed in over 90% of the cases. We have previously shown that PMX preadministration had the same effect against human ovarian cancer (IGROV-1 and SKOV-3) xenografted mice.[24] However, all of those cell lines or xenografts, respectively, express the human FR-α which is slightly different from the mouse FR that is expressed in the kidneys of the host mice. One could have assumed that species differences of FRs in the tumor xenografts and kidneys, respectively, would feature different affinities for PMX and thus result in a different interaction of PMX and the radiofolate in these tissues. To disprove this hypothesis we tested the effect in a syngeneic tumor mouse model that expresses murine FR. The M109 tumor model is based on a FR-positive syngeneic lung adenocarcinoma graft. Incidentally, M109 tumors express similar numbers of receptors as many human ovarian carcinomas and thus, represent a clinically valuable model when grafted to mice for investigations of folic acid-targeted diagnostics and therapeutics.[17,34,35] Tissue distribution data of ^{99m}Tc-EC20 were largely com-

(32) Zhao, R. B.; Goldman, I. D. The molecular identity and characterization of a proton-coupled folate transporter-PCFT; biological ramifications and impact on the activity of pemetrexed. *Cancer Metastasis Rev.* **2007**, *26* (1), 129–139.

(33) Mauritz, R.; Peters, G. J.; Kathmann, I.; Teshale, H.; Noordhuis, P.; Comijn, E. M.; Pinedo, H. M.; Jansen, G. Dynamics of antifolate transport via the reduced folate carrier and the membrane folate receptor in murine leukaemia cells in vitro and in vivo. *Cancer Chemother. Pharmacol.* **2008**, *62* (6), 937–48.

(34) Leamon, C. P.; Reddy, J. A.; Vlahov, I. R.; Westrick, E.; Parker, N.; Nicoson, J. S.; Vetzel, M. Comparative preclinical activity of the folate-targeted Vinca alkaloid conjugates EC140 and EC145. *Int. J. Cancer* **2007**, *121* (7), 1585–92.

parable in mice bearing KB-tumors and those with M109 tumor grafts. Importantly, we found that PMX application resulted in the same effect in the syngeneic tumor model than in KB xenografted mice indicating that species differences of the receptors in tumors and kidneys were not the reason for our observation.

In summary, we were able to show that the effect of the antifolate PMX in combination with radiofolates is unique and not reproducible with an FR-selective antifolate, such as CB3717. Kidney uptake of radioactivity was significantly reduced after injection of PMX without affecting the tumor uptake independent of whether the tumor was a human xenograft or a syngeneic murine graft. This effect is of particular interest in view of the development of FR-targeted radionuclide therapy because a high radiation dose to the kidneys would be problematic due to the risk of renal damage. Given that background radiation in other organs and tissues is negligible, it is generally accepted that a tumor-to-kidney uptake ratio of >1 would allow for radionuclide therapy studies. In the study presented herein it was shown that the tumor-to-kidney ratio reached a value of 1.2−1.3 at 4 and 24 h p.i. when PMX was applied at a sufficient dose and combined with ^{99m}Tc-EC20. In this study, we used ^{99m}Tc-EC20 because it is currently the only clinically used radiofolate. Obviously, the design of any future radiotherapy study would have to be adapted to the properties of a folate conjugate labeled with a suitable therapeutic radionuclide.

Acknowledgment. The authors wish to thank Alicia Bloomfield, Melissa Nelson, Ryan Dorton, and Le-cun Xu for their expert technical assistance with EC20 sample preparation and help with the tissue biodistribution studies (M109 model).

MP900296K

(35) Leamon, C. P.; Reddy, J. A.; Vlahov, I. R.; Westrick, E.; Dawson, A.; Dorton, R.; Vetzel, M.; Santhapuram, H. K.; Wang, Y. Preclinical antitumor activity of a novel folate-targeted dual drug conjugate. *Mol. Pharmacol.* **2007**, *4* (5), 659–67.