Supporting Information

Radioiodinated Folic Acid Conjugates: Evaluation of a Valuable Concept to Improve Tumor-to-Background Contrast

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1. Organic Syntheses and Radioiodination

Experimental. All chemicals were purchased from Sigma-Aldrich/Fluka, Buchs, Switzerland or Bachem, Dübendorf, Switzerland, except 2-(1H-Benotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophophate (HBTU), which was purchased from Novabiochem, Darmstadt, Germany. Protected pteroic acid (N^2-*N*,*N*-dimethylaminomethylene-10-formyl-pteroic acid) was kindly provided by Merck & Cie, Schaffhausen, Switzerland. All chemicals and solvents were of reagent grade and were used without further purification. Reactions were monitored by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Sep-Pak™ columns (Waters) were washed with methanol (MeOH) and water prior to use. Products were eluted using water or a water/MeOH gradient. Nuclear magnetic resonance spectra were recorded on a 400 MHz Bruker spectrometer. LC/MS analysis was performed on a Waters LCT Premier mass spectrometer and analysis was performed using *MassLynx* (version 4.1) software.

For analysis and purification of non-radiolabeled and radioiodinated products, an HPLC system (Merck Hitachi L-6200A-system) was used that was equipped with a tunable absorption detector and a radiometric detector using a C-18 reversed-phase column (Waters, XTerra™, MS, C-18, 5 µm, 4.6 x 150 mm). The eluent system consisted of MeOH and triethylammonium phosphate buffer (TEAP) pH 4.4 with a gradient from 5% to 80% MeOH over 25 min with a flow rate of 1 ml/min.

The structures of the folate conjugates are shown in Figure 1. The synthesis of compound **1** is outlined in Figure S1. Boc-Glu-OMe (1.5 equivalents) and HBTU (1.5 equivalents) were dissolved in dimethylformamide (DMF, 5 mL/mmol). Triethylamine (TEA, 3 equivalents) was added and the resulting solution was stirred at room temperature for 30 minutes. This solution was added slowly to a stirring solution of the H-Tyr(O^tBu)-OMe (**S1**, 1 equivalent) dissolved in a mixture of DMF (5 mL/mmol) and TEA (3 equivalents) and stirred at room temperature. After two hours, the product (**S2**) was extracted into ethyl acetate (EtOAc), and washed with water and brine. The organic phase was dried over sodium sulfate and after removal of the solvent under reduced pressure the crude product was purified by column chromatography with mixtures of dichloromethane (DCM) and MeOH. The Boc and t-butyl protecting groups were removed in a 10:1 mixture of DCM and trifluoroacetic acid (TFA). N^2-*N*,*N*-dimethylaminomethylene-10-formyl-pteroic acid and H-Glu(Tyr(OMe))-OMe (**S3**) were coupled and purified using the procedure described above. The product (**S4**) was deprotected in a 1:1 mixture of 1M NaOH and MeOH. The neutralized reaction solution was purified using a Sep-Pak™ column. Fractions containing

the product (**1**) were combined and after reducing the volume of solvent, the HCl salt of the product was precipitated with 1M HCl and collected by centrifugation.

Compound **2** was synthesized from commercially available 4-iodo-tyrosine, which was first converted to its methyl ester using trimethylsilyl chloride (TMSCl) in MeOH. Coupling to Boc-Glu-OMe and to the N^2-*N,N*-dimethylaminomethylene-10-formyl-pteroic acid derivative, as well as deprotection procedures were carried out as described above. Compound **2** was purified by semi-preparative HPLC using a C-18 reversed phase column (Waters, XBridge™, C-18, 5 µm, 10 x 150mm) and an eluent system consisting of MeOH and a NH_4HCO_3 solution (50 mM, pH 8) with a gradient from 0% to 33% MeOH over 21 min with a flow rate of 3.5 ml/min.

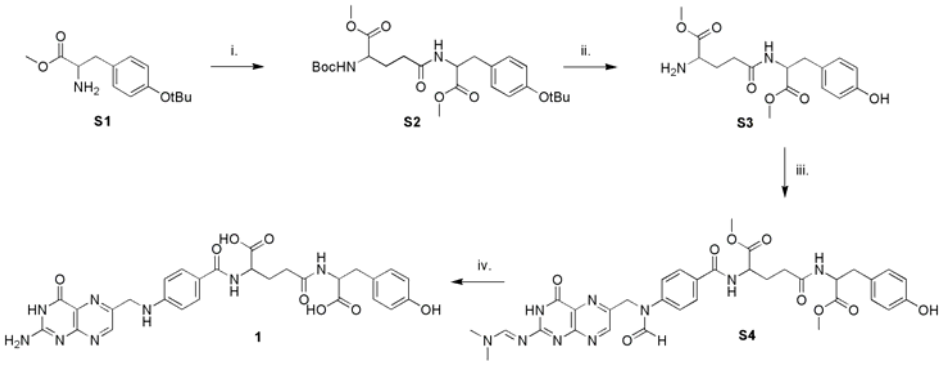

Figure S1. Synthesis of compound **1**: (i) Boc-Glu-OMe, HBTU, DMF, TEA; (ii) DCM/TFA; (iii) N^2-*N,N*-dimethylaminomethylene-10-formyl-pteroic acid, HBTU, DMF, TEA; (iv) 1 M NaOH/MeOH; followed by 1 M HCl.

Compounds **3** and **4** were synthesized using Cu(I)-catalyzed cycloaddition reactions (referred to as a "click reaction") of an azido-folic acid derivative and propargyl-functionalized tyrosine derivatives (Fig. S2). The azido-folic acid derivative was prepared according to a previously published procedure.[1] H-Tyr(O^tBu)-OMe (**S5**) and H-4-iodo-Tyr-OMe were alkylated with 3-bromo-propyne (1.5 equivalents) and Cs_2CO_3 (1.5 equivalents) in DMF at room temperature. The alkylated products (**S6**) were extracted into EtOAc, and washed with water and brine. The organic phases were dried over sodium sulfate and after removal of the solvent under reduced pressure the crude products were purified by column chromatography with mixtures of DCM and MeOH. For the synthesis of compound **3**, the tert-butyl protecting group was removed in a 10:1 mixture of DCM and TFA, after which the alkyne derivative (**S7**) was purified using a Sep-Pak™ column. For both compounds **3**

and **4,** the cycloaddition reactions were carried out at room temperature with one equivalent of the alkyne component and one equivalent of azido-folic acid in a 1:1 mixture of water and tert-BuOH. NaOH (1M) was added drop-wise until the solutions were clear, followed by copper acetate (0.1 equivalents) and sodium ascorbate (0.2 equivalents) and the reactions were stirred for 12 hours. The neutralized reaction mixtures were purified using Sep-Pak^{TM} columns. Fractions containing the product were combined and after reducing the volume of solvent, the HCl salts of the products (**3** or **4**) were precipitated with 1M HCl and collected by centrifugation.

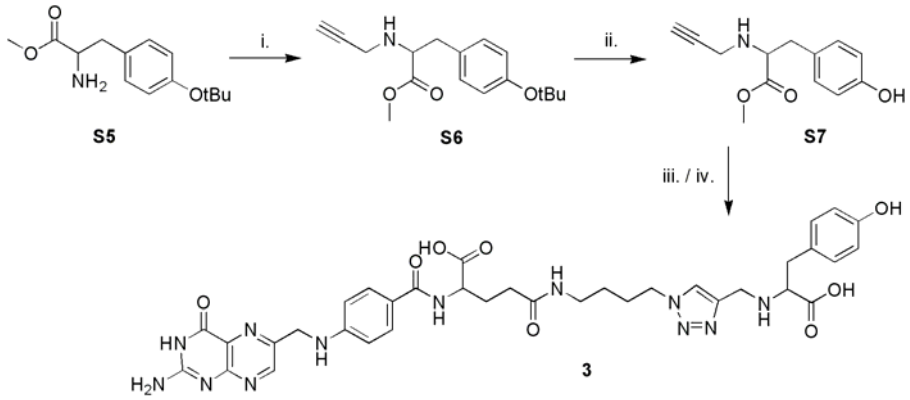

Figure S2. Synthesis of compound **3**: (i) 3-bromo-propyne, Cs_2CO_3, DMF; (ii) DCM/TFA; (iii) azido-folic acid, copper acetate, sodium ascorbate, H_2O/tert-BuOH/NaOH; (iv)1M HCl.

Compounds **1** and **3** were radiolabeled with [^{125}I]-iodine and [^{131}I]-iodine isotopes obtained from Perkin Elmer (Waltham, MA, USA) to obtain [^{125}I]-**2** and [$^{125/131}$I]-**4**.

Results and Conclusion of Organic Syntheses and Radioiodination. Syntheses of the tyrosine-folate (**1**) and iodo-tyrosine-folate (**2**) resulted in an overall yield of ~ 10% for compound **1** and ~ 5% of compound **2**. The conjugation of the tyrosine moiety using "click chemistry" was straightforward and resulted in an overall yield of ~ 25% for tyrosine-click-folate (**3**) and ~ 14% for iodo-tyrosine-click-folate (**4**). All compounds were obtained in > 95% purity as confirmed by HPLC. Characterization data obtained by LC/MS electrospray ionization (ESI) are shown in Table S1.

Table S1. Calculated and Observed Masses from LC/MS

Compound	Determined Mass	Sum Formula	Calculated Mass [g/mol]	Found Mass [g/mol]
1	$[M+H]^+$	$C_{28}H_{29}N_8O_8$	605.21	605.21
2	$[M+H]^+$	$C_{28}H_{28}IN_8O_8$	731.11	731.10
3	$[M-H+2Na]^+$	$C_{35}H_{38}N_{12}O_8Na_2$	801.28	801.28
4	$[M+H]^+$	$C_{35}H_{40}IN_{12}O_8$	883.21	883.21

After radioiodination, identification and quality control of [^{125}I]-**2** and [$^{125/131}$I]-**4** were carried out by comparison of the retention times with the non-radioactive reference compounds **2** and **4**. Compounds [^{125}I]-**2** and [$^{125/131}$I]-**4** were separated from traces of free iodine and non-radioactive precursor **1** or **3**, respectively, by HPLC (Fig. S3).

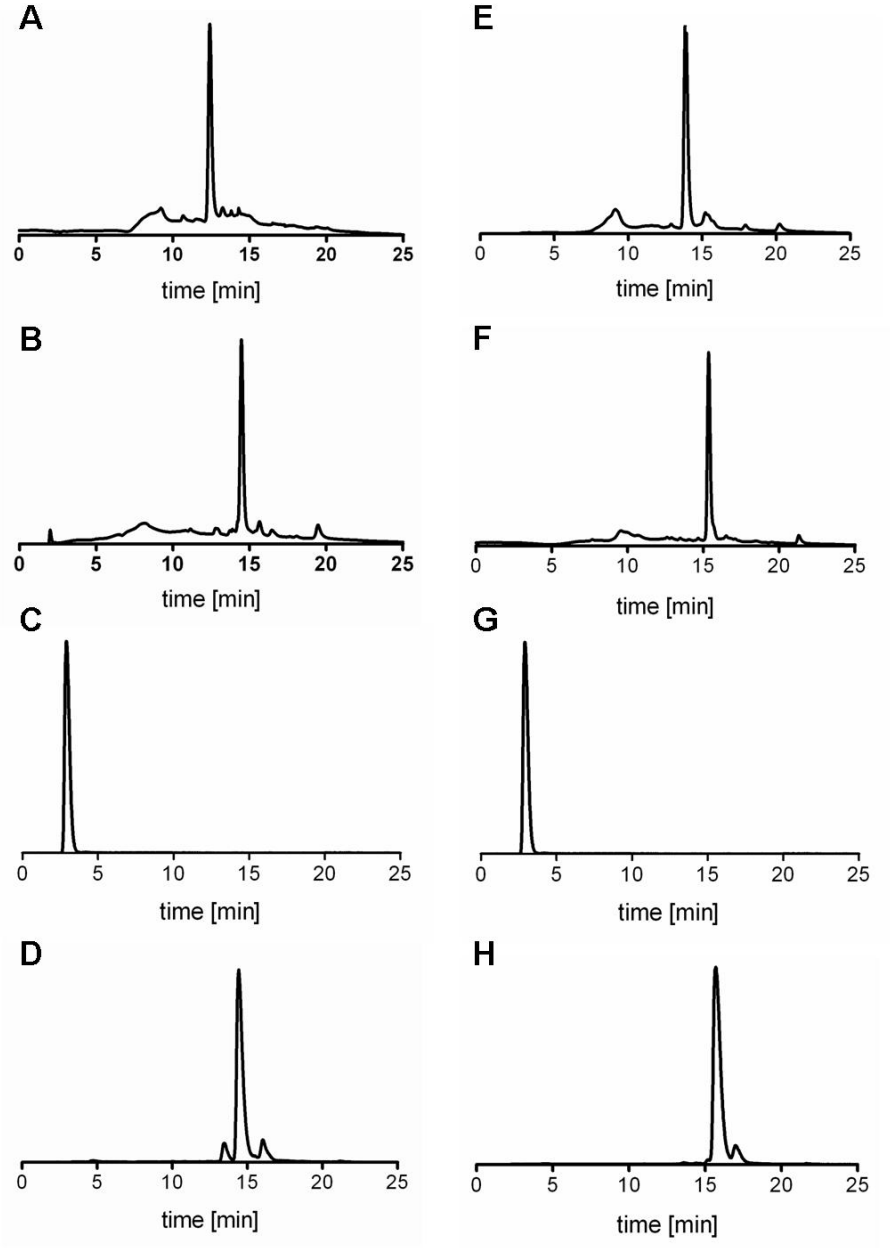

Figure S3. HPLC UV traces (254 nm) of (A) tyrosine-folate (**1**: $R_t \approx 12.4$ min); (B) iodo-tyrosine-folate (**2**: $R_t \approx 14.5$ min); (E) tyrosine-click-folate (**3**: $R_t \approx 13.9$ min) and (F) iodo-tyrosine-click-folate (**4**: $R_t \approx 15.4$ min). HPLC γ traces of (C and G) free [^{125}I]-iodide ($R_t \approx 2.9$ min); (D) ^{125}I-radioiodinated tyrosine-folate ([^{125}I]-**2**: $R_t \approx 14.4$ min) and (H) ^{125}I-radioiodinated tyrosine-click-folate ([^{125}I]-**4**]: $R_t \approx 15.7$ min).

2. Octanol/PBS Distribution Coefficient (LogD)

Experimental. The distribution coefficient (log D) of both radiofolates was determined by a previously published procedure.[2,3] In brief, a sample of the HPLC-purified radiofolate (600 kBq) was mixed with equal amounts of PBS pH 7.4 and octanol (1500 µl each). The vials were vortexed vigorously for 1 min. To achieve phase separation, the vials were centrifuged (2500 rpm) for 6 min. The radioactivity concentration was determined in a defined volume of each layer measured in a γ-counter. The distribution coefficient was expressed as the ratio of counts per minute (cpm) measured in the octanol phase to the cpm measured in the PBS phase. The results represent the mean of three independent measurements (± SD), each performed in quintuplicate.

Results and Conclusion of the Determination of the Octanol/PBS Distribution Coefficient (LogD). The log D value obtained for [^{125}I]-**2** was slightly higher (-2.87 ± 0.02) than for [^{125}I]-**4** (-3.13 ± 0.07) indicating that ^{125}I-tyrosine-click-folate ([^{125}I]-**4**) is more hydrophilic than ^{125}I-tyrosine-folate ([^{125}I]-**2**). The relatively lipophilic character of both radiofolates is comparable to previously evaluated ^{99m}Tc(CO)$_3$-folate conjugates (^{99m}Tc(CO)$_3$-His-folate:[4] log D = - 3.47 ± 0.05 and ^{99m}Tc(CO)$_3$-PAMA-folate:[5, 6] log D = - 2.12 ± 0.01) and in contrast to the notably more hydrophilic character of e.g. ^{111}In-DOTA-folate (log D = - 4.21 ± 0.11).[3] The relatively lipophilic properties of [^{125}I]-**2** and [^{125}I]-**4** are responsible for undesired unspecific accumulation of radioactivity in the intestinal tract as has previously been observed with ^{99m}Tc(CO)$_3$-folate conjugates.[4, 6] The pharmacokinetic properties of radioiodinated folates might be improved by incorporating more hydrophilic spacer entities to achieve logD values in the range of those of DOTA-folate conjugates.

3. Investigation of Metabolic Stability Using Liver Microsomes

Experimental. Metabolic stability of [^{125}I]-**2** (R_t ≈ 14.4 min) and [^{125}I]-**4** (R_t ≈ 15.7 min) was investigated in vitro using murine liver microsomes (BD Biosciences, Cat.-N° 452702) which contain a series of enzymes which may be responsible for in vivo deiodination processes. The experiments were performed according to the manufacturer's instruction using liver microsomes at a concentration of 0.5 mg/mL in potassium phosphate buffer pH 7.4. A 5:1 mixture of solution A (31 mM $NADP^+$, 66 mM glucose-6-phosphate, 66 mM $MgCl_2$ in water; Cat.-N° 451220) and solution B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, Cat.-N° 451200) was used as NADPH regenerating system for reactions of cytochrome P450. Test compounds [^{125}I]-**2** and [^{125}I]-**4** were added

in a volume of 15 µL with a radioactivity of ~ 4 MBq. Control experiments were performed by incubation of the radiofolates [^{125}I]-**2** and [^{125}I]-**4** with the regenerating solutions in buffer without microsomes. In addition, ^{125}I-iodinated L-tyrosine ($R_t \approx 8.6$ min) was investigated under the same experimental conditions. After an incubation time of 0 min, 30 min, 4 h and 24 h proteins were precipitated by addition of 200 µL cold MeOH to each sample. After centrifugation the supernatants were analyzed using HPLC and deiodination was quantified by integration of the peak which corresponded to free iodide ($R_t = 2.9$ min).

Results and Conclusion of in Vitro Stability Experiments Using Liver Microsomes. Experiments performed with liver microsomes revealed a fast deiodination of [^{125}I]iodo-L-tyrosine. After an incubation time of 30 min at 37°C, 90% of [^{125}I]iodo-L-tyrosine was deiodinated (Fig. S4 A & B). In contrast, deiodination was not observed for [^{125}I]-**2** (Fig. S4 C & D) and [^{125}I]-**4** (Fig. S4 E & F) after the same time period of incubation with liver microsomes. Even after longer incubation times (4 h and 24 h) of [^{125}I]-**2** and [^{125}I]-**4** with liver microsomes free iodide was not detected (data not shown) indicating that [^{125}I]-**2** and [^{125}I]-**4** were stable under these experimental conditions.

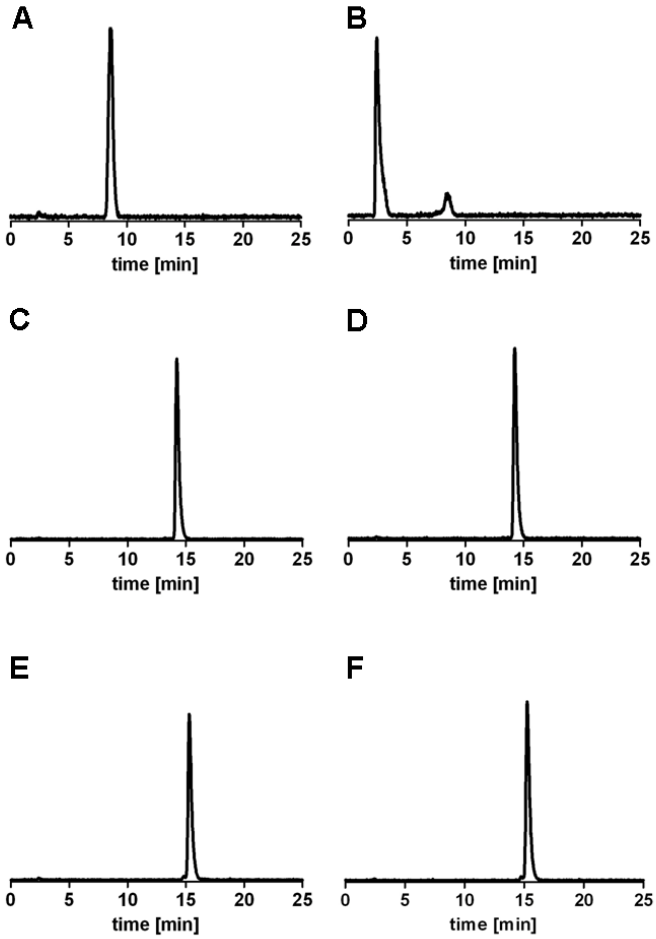

Figure S4. HPLC traces of (A) [^{125}I]iodo-L-tyrosine (R_t ≈ 8.6 min); (B) supernatant after incubation of [^{125}I]iodo-L-tyrosine with liver microsomes for 30 min (free [^{125}I]-iodide: R_t ≈ 2.9 min); (C) [^{125}I]-**2**: R_t ≈ 14.4 min; (D) supernatant after incubation of [^{125}I]-**2** with liver microsomes for 30 min; (E) [^{125}I]-**4**: R_t ≈ 15.7 min; (F) supernatant after incubation of [^{125}I]-**4** with liver microsomes for 30 min.

4. Metabolite Studies

Experimental. Normal six to eight-week-old female Balb/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were fed with a folate-deficient rodent diet starting 5 days before the experiments.[7] All mice received an intraperitoneal injection of potassium iodide (4 mg in 200 µl PBS pH 7.4) 1 h before an intravenous injection of ~ 5 MBq (~ 64 pmol) ^{125}I-radioiodinated compound [^{125}I]-**2** or

[^{125}I]-**4**. Urine and blood samples were taken 5 min and 30 min after injection of the radiotracer (n = 2 per time point). Immediately afterwards, the mice were euthanized by cervical dislocation under anesthesia. Plasma samples (~ 50 µL) were prepared by centrifugation (10 min, 1.6 rpm) of the blood collected in heparinized vials. Plasma proteins were precipitated by addition of 200 µL MeOH. After centrifugation, the supernatants were analyzed by HPLC. Urine samples were directly injected into the HPLC. Peaks on the HPLC spectra representing the intact radiofolate [^{125}I]-**2** or [^{125}I]-**4**, radioactive metabolites of unknown composition, and of free iodide were integrated and quantified.

Results and Conclusion of Metabolite Studies. The analysis of blood samples confirmed a higher grade of deiodination of compound [^{125}I]-**2** compared to compound [^{125}I]-**4**. As a consequence a high concentration of free [^{125}I]-iodide was found in the blood of mice injected with [^{125}I]-**2** but was not detected in blood samples of mice that received compound [^{125}I]-**4** (Table S2). Excretion of released [^{125}I]-iodide via urine was monitored for both compounds and further confirmed quick degradation of compound [^{125}I]-**2**. Intact radiofolate [^{125}I]-**2** was not detectable in urine samples. In contrast, almost the whole radioactive fraction in urine samples taken from mice 5 min after injection of [^{125}I]-**4** corresponded to the intact radiofolate [^{125}I]-**4** and only a small amount (~ 10%) to free [^{125}I]-iodide (Table S2).

Table S2. HPLC Analysis of Blood and Urine Samples from Mice Injected with Radiofolate [^{125}I]-**2** and [^{125}I]-**4**

^{125}I-Tyrosine-Folate [^{125}I]-**2**						
	urine			blood		
time p.i.	free ^{125}I$^-$	metabolite	intact [^{125}I]-**2**	free ^{125}I$^-$	metabolite	intact [^{125}I]-**2**
5 min	n.d.	n.d.	n.d.	10%	10%	80%
30 min	100%	-	-	90%	-	10%
^{125}I-Tyrosine-Click-Folate [^{125}I]-**4**						
	urine			blood		
time p.i.	free ^{125}I$^-$	metabolite	intact [^{125}I]-**4**	free ^{125}I$^-$	metabolite	intact [^{125}I]-**4**
5 min	10%	-	90%	-	-	100%
30 min	70%	15%	5%	n.d.	n.d.	n.d.

5. Biodistribution of [^{125}I]-4 in Combination with Pemetrexed

Experimental. Six to eight-week-old female, athymic nude mice (CD-1 Foxn-1/nu) were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were fed with a folate-deficient rodent diet (Harlan Laboratories, U.S.) starting 5 days prior to tumor cell inoculation.[7] Mice were inoculated with KB cells (5 x 10^6 cells in 100 µl PBS) into the subcutis of each shoulder. Animal experiments were performed approximately 14 days after tumor cell inoculation. Biodistribution studies were performed in triplicate. Pemetrexed (PMX) was diluted with NaCl 0.9% according to the instructions of the manufacturer and injected into a lateral tail vein (400 µg in 100 µl), 1 h prior to the radiotracer.[8, 9] Non-radioactive potassium iodide was dissolved in PBS pH 7.4 and injected intraperitoneally (4 mg in 200 µl), 1 h prior to radioactivity. The effect of different doses of PMX pre-administration was investigated in combination with the ^{125}I-tyrosine-click-folate ([^{125}I]-**4**) at 4 h p.i. of the radiotracer

Results and Conclusion of the Biodistribution of [^{125}I]-4 in Combination with Pemetrexed. Previously it was demonstrated that the uptake of radiofolates in the kidneys of mice is significantly reduced if PMX is injected prior to the radiofolate.[3, 8-10] Notably, PMX has no significant influence on radiofolate uptake in FR positive tumor (xeno)grafts giving rise to unprecedentedly high tumor-to-kidney ratios of radioactivity in these mice. This approach was also tested in combination with our novel radioiodinated tyrosine-click-folate tracer [^{125}I]-**4**. Biodistribution data obtained with variable doses of pre-injected PMX are shown in Table 2. The tumor uptake was slightly reduced after administration of the highest dose (400 µg) of PMX (1.15 ± 0.17%; $p = 0.08$) whereas almost no tumor reduction was observed after administration of 50% of the PMX dose ($p = 0.64$) and 25% of the PMX dose ($p = 0.19$). In contrast, kidney accumulation of radioactivity was significantly reduced after administration of PMX in a dose-dependent manner (400 µg, $p = 0.01$, 200 µg, $p = 0.01$, 100 µg, $p = 0.01$). Thus, by employing the highest dose of PMX, the tumor-to-kidney ratio could be improved > 10-fold. These data confirmed our previous findings obtained with ^{99m}Tc-radiolabeled folate conjugates in combination with PMX.[8, 11] As expected, tumor-to-kidney ratios were significantly improved, however, no significant changes in the tumor-to-blood, tumor-to-liver, or tumor-to-muscle ratios were observed in mice that had received PMX (Fig. S5).

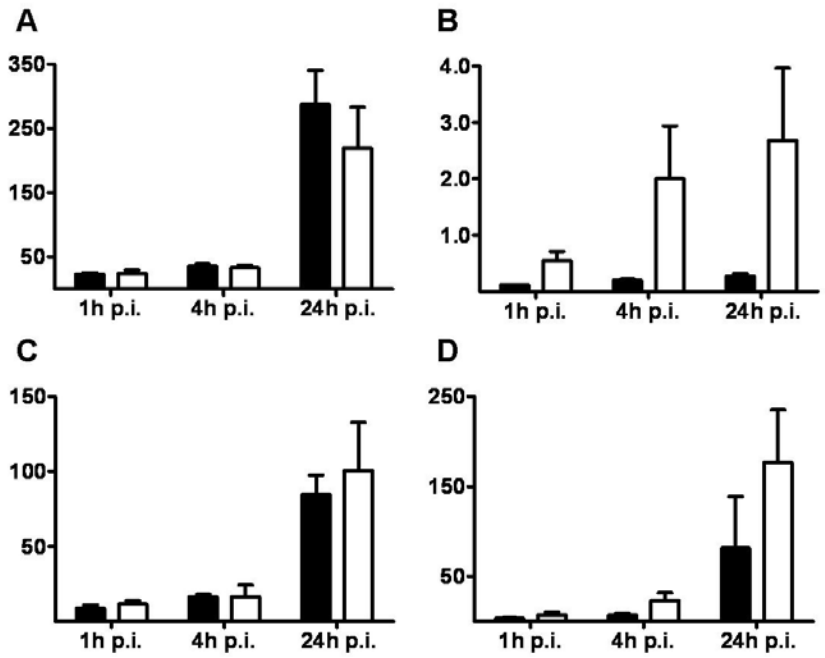

Figure S5. Tumor-to-background ratios of radioactivity 4 h after injection of [^{125}I]-**4**: tumor-to-blood (A), tumor-to-kidney (B), tumor-to-liver (C) and tumor-to-muscle ratios (D). Values from control mice are shown in black and values from mice pre-injected with PMX (200 μg) are shown in white.

6. Ex Vivo Autoradiography

Experimental. For autoradiography studies, mice were injected with radiofolate [^{125}I]-**4** (~ 2.0 MBq, ~ 25 pmol) with or without pre-injected PMX (400 µg) and sacrificed ~ 24 h after injection. Immediately after euthanasia, tumors and kidneys were collected, embedded in TissueTek (O.C.T. Compount; Sakura Finetek Europa B.V) and frozen at -80°C. Frozen tissues and organs were cut into 10 µm sections with a cryomicrotome (Model OFT, Bright Instrument Co Ltd, England) and mounted on slides (Superfrost plus, Menzel). The slides were exposed to phosphor imaging screens (Super Resolution Type SR, Perkin Elmer) for 2.5 h in x-ray cassettes. The screens were then read by a phosphor imager (Cyclone Plus phosphor imager, PerkinElmer). Quantification of radioactivity uptake in tumors and kidneys was carried out by analyzing the intensity of blackening [DLU/mm^2] of several sections from the same tissue and expressed as average values. Average DLU/mm^2-values obtained for tumors and kidneys of a control mouse were defined as 100%. Quantitative analysis and preparation of images were carried out using the software *OptiQuant* (version 5.0) and *Adobe Photoshop* (version 10).

Results and Conclusion of Ex Vivo Autoradiography. Images of KB tumor sections and kidneys from a control mouse and a mouse that received PMX are shown in Figure 8. In tumor sections of the mouse that received PMX quantification showed that the intensity was reduced to 56% of the intensity obtained in control tumor sections. Radioactivity uptake in the kidneys of the control mouse was high and localized primarily in the renal cortex. Quantification of radioactivity in kidney sections of the mouse that received PMX showed that the uptake had been reduced to less than 4% of the value obtained for kidney sections from a control mouse. These findings confirm the data obtained from the biodistribution study (Table 2) and provide additional information about precise localization of the conjugates within these FR positive KB tumors and kidneys.

7. References

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