

Supplemental *in vivo* data

Biodistribution data of ^{99m}Tc -PAMA-folate as function of time of pre-application of antifolates

Data in supplemental Table 1 complement the results presented in Figure 2 of the manuscript.

SUPPLEMENTAL TABLE 1

Biodistribution (% ID/g, 4h p.i.) of the ^{99m}Tc -PAMA-folate radiotracer depending on the time of pre-administered antifolates.

	Methotrexate^{c*}					
	3d ^a	24 h ^b	2 h ^b	1 h ^b	30 min ^b	15 min ^b
blood	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.09 ± 0.08
heart	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.06 ± 0.09
lung	0.02 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.08 ± 0.11	0.14 ± 0.14
spleen	0.10 ± 0.12	0.06 ± 0.02	0.03 ± 0.01	0.04 ± 0.04	0.05 ± 0.07	0.07 ± 0.02
kidney	8.98 ± 2.57	9.35 ± 1.73	5.15 ± 0.38	2.45 ± 1.32	1.03 ± 0.20	0.73 ± 0.24
stomach	1.38 ± 0.80	0.25 ± 0.20	0.37 ± 0.29	0.09 ± 0.04	0.19 ± 0.06	0.40 ± 0.28
intestines	7.92 ± 7.65	1.16 ± 0.13	1.78 ± 0.43	0.90 ± 0.71	0.52 ± 0.14	0.54 ± 0.23
contents of intestines	19.60 ± 34.01	4.29 ± 4.05	7.79 ± 1.22	9.37 ± 10.27	4.14 ± 1.65	4.16 ± 3.55
liver	8.48 ± 6.92	2.99 ± 4.18	3.71 ± 3.12	0.85 ± 0.79	1.07 ± 1.23	0.35 ± 0.09
muscle	0.07 ± 0.03	0.07 ± 0.01	0.09 ± 0.03	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
bone	0.03 ± 0.02	0.02 ± 0.00	0.06 ± 0.04	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.01
tumor	0.71 ± 0.32	1.35 ± 0.33	0.91 ± 0.25	1.41 ± 0.65	0.63 ± 0.02	0.58 ± 0.12
tumor-to-blood	33.37 ± 19.53	62.40 ± 30.42	42.43 ± 8.04	133.9 ± 46.8	57.16 ± 7.37	23.58 ± 32.11
tumor-to-liver	0.34 ± 0.51	2.15 ± 2.45	0.87 ± 1.24	2.61 ± 1.81	1.30 ± 1.04	1.83 ± 0.92
tumor-to-kidney	0.08 ± 0.02	0.15 ± 0.03	0.18 ± 0.04	0.59 ± 0.12	0.62 ± 0.14	0.87 ± 0.38
	Raltitrexed^{c,*}					
blood			0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.05	0.01 ± 0.00
heart			0.07 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
lung			0.10 ± 0.02	0.04 ± 0.01	0.07 ± 0.02	0.04 ± 0.02

spleen	0.06 ± 0.05	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.02
kidney	8.23 ± 2.93	3.08 ± 0.28	4.10 ± 1.71	2.63 ± 1.29
stomach	0.32 ± 0.29	0.06 ± 0.03	0.40 ± 0.42	0.11 ± 0.06
intestines	0.74 ± 0.20	1.13 ± 1.27	1.01 ± 0.28	0.63 ± 0.28
contents of intestines	6.07 ± 2.17	14.58 ± 17.85	10.28 ± 3.36	8.56 ± 9.04
liver	1.21 ± 0.73	0.47 ± 0.36	2.70 ± 2.14	1.53 ± 0.37
muscle	0.15 ± 0.03	0.04 ± 0.03	0.07 ± 0.03	0.05 ± 0.02
bone	0.05 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.02
tumor	1.19 ± 0.32	1.07 ± 0.09	1.39 ± 0.15	1.15 ± 0.38
tumor-to-blood	54.07 ± 22.13	84.52 ± 28.27	66.88 ± 54.12	120.77 ± 43.10
tumor-to-liver	1.57 ± 1.54	4.88 ± 5.52	0.89 ± 0.81	0.74 ± 0.07
tumor-to-kidney	0.16 ± 0.09	0.35 ± 0.05	0.38 ± 0.15	0.47 ± 0.12
Pemetrexed^{d,*}				
blood	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.08 ± 0.01
heart	0.19 ± 0.01	0.09 ± 0.07	0.07 ± 0.01	0.13 ± 0.01
lung	0.87 ± 1.14	0.19 ± 0.08	0.06 ± 0.01	0.14 ± 0.03
spleen	0.23 ± 0.27	0.39 ± 0.55	0.06 ± 0.07	0.04 ± 0.01
kidney	2.03 ± 0.27	1.14 ± 0.18	0.50 ± 0.17	0.90 ± 0.18
stomach	0.64 ± 0.54	5.85 ± 9.27	0.14 ± 0.11	0.24 ± 0.07
intestines	1.25 ± 0.27	1.34 ± 0.20	1.10 ± 1.09	1.18 ± 0.33
contents of intestines	7.69 ± 2.83	21.31 ± 18.25	10.16 ± 7.96	4.56 ± 2.81
liver	1.57 ± 1.01	0.97 ± 0.25	1.65 ± 0.67	5.31 ± 3.53
muscle	0.31 ± 0.06	0.22 ± 0.04	0.13 ± 0.06	0.11 ± 0.02
bone	0.19 ± 0.10	0.09 ± 0.03	0.02 ± 0.01	0.16 ± 0.15
tumor	1.52 ± 0.05	2.21 ± 0.34	0.93 ± 0.45	0.98 ± 0.15
tumor-to-blood	56.15 ± 11.14	82.07 ± 4.39	96.62 ± 45.60	13.42 ± 3.79
tumor-to-liver	1.61 ± 1.52	2.38 ± 0.73	0.60 ± 0.27	0.25 ± 0.14
tumor-to-kidney	0.76 ± 0.13	1.99 ± 0.51	1.80 ± 0.27	1.11 ± 0.22

^a via drinking water; 80 µg/mL

^b pre-injected, 400 µg/mouse

^c pre-injected, 100 µg/mouse

^d pre-injected, 400 µg/mouse

* n = 3

Biodistribution data of ^{99m}Tc-PAMA-folate in leucovorin pre-injected mice

Data in supplemental Table 2 complement the results described in paragraph “*Effect of intravenously administered LV*” of the manuscript.

SUPPLEMENTAL TABLE 2

Biodistribution (% ID/g, 4h p.i.) of the ^{99m}Tc-PAMA-folate radiotracer in KB-tumor bearing athymic nude mice, i.v. injected with leucovorin (LV) 1 h previous to the radiotracer (in comparison with control data).

	Leucovorin^a	Control
blood	0.01 ± 0.00	0.04 ± 0.00
heart	0.01 ± 0.00	0.32 ± 0.07
lung	0.01 ± 0.00	0.33 ± 0.02
spleen	0.00 ± 0.00	0.15 ± 0.04
kidney	0.24 ± 0.09	18.48 ± 0.72
stomach	0.26 ± 0.33	0.63 ± 0.33
intestines	0.93 ± 1.00	1.49 ± 0.23
contents of intestines	14.89 ± 21.21	38.51 ± 55.88
liver	1.72 ± 1.84	2.37 ± 2.85
muscle	0.01 ± 0.01	0.54 ± 0.05
bone	0.00 ± 0.00	0.31 ± 0.07
tumor	0.20 ± 0.04	2.33 ± 0.36
tumor-to-blood	30.73 ± 7.47	58.0 ± 12.2
tumor-to-liver	0.21 ± 0.14	2.53 ± 2.13
tumor-to-kidney	0.87 ± 0.24	0.13 ± 0.02

^a pre-injected, 100 µg/mouse

Supplemental *in vitro* data

Time dependent effect of pre-incubation of KB-cells with antifolates MTX, RTX, PMX and LV

Purpose: The influence of time of pre-incubation of KB-cancer cell lines with methotrexate (MTX), raltitrexed (RTX), pemetrexed (PMX) and reduced folate leucovorin (LV) on the cellular uptake of ^{99m}Tc -PAMA-folate was investigated. These *in vitro* experiments should provide evidence that short exposure of the cells to antifolates or the reduced folate LV (1 h) does not have any influence on the radiotracer uptake or the concentration of α -FR expression level on the cells. The 1 hour time point was chosen, because *in vivo* experiments with pre-injection of tumor bearing mice with MTX, RTX and PMX gave optimal tumor-to-kidney ratios.

Experimental Design and Results: The *in vitro* experiments were performed with cells, seeded in 12-well plates (8×10^5 cells in 2 mL/well), which had been incubated at 37°C overnight to form confluent monolayers. One hour prior to the experiment, the culture medium was removed and the cells were incubated with FFRPMI containing the antifolates (MTX, RTX and PMX) or the reduced folate leucovorin (LV) (10.0 μM ; 2 mL/well) at 37°C. Then, the supernatants with antifolates or LV were removed from each well. The KB cell monolayers were rinsed twice with ice-cold PBS (pH 7.4). Pure FFRPMI medium (without FCS/L-glutamine/antibiotics, 975 μL) was added into each well. The well plates were pre-incubated at 37°C for 10 min. A solution of the ^{99m}Tc -PAMA-folate (25 μL , 1 MBq/mL) was added and the well plates were incubated at 37°C for 1 h. Then, the supernatants were removed and the monolayers washed with ice-cold PBS. In order to release the radiotracer from FRs on the cell surface and to determine the internalized fraction the cell samples were washed using an acidic stripping buffer (aqueous solution of 0.1 M acetic acid and 0.15 M NaCl, pH 3). The cells were lysed in 1N NaOH (1 mL), transferred in 4 mL tubes and counted for radioactivity using a γ -counter. The results, such as cellular uptake and internalized fraction of the radiotracer were calculated as percentage of total added radioactivity and presented in supplemental Table 3

SUPPLEMENTAL TABLE 3

Total binding (uptake) and internalized fraction (acid-resistant) of the ^{99m}Tc -PAMA-folate radiotracer after incubation of the KB-cells with antifolates and leucovorin for 1 h and for 24 hours.

	Methotrexate		Raltitrexed		Pemetrexed		Leucovorin	
time of pre-incubation*	total binding (%)	internalization (%)	total binding (%)	internalization (%)	total binding (%)	internalization (%)	total binding (%)	internalization (%)
Control**	31.8 ± 7.0	4.5 ± 0.9	25.7 ± 0.6	7.8 ± 0.5	34.6 ± 1.4	10.2 ± 1.2	33.1 ± 1.4	6.8 ± 0.8
1 hour	26.9 ± 1.80	8.1 ± 0.87	26.9 ± 1.78	5.7 ± 0.60	24.8 ± 0.82	4.9 ± 0.91	24.4 ± 0.77	6.5 ± 0.86
24 hours**	77.3 ± 5.7	18.8 ± 1.8	64.5 ± 3.5	19.0 ± 2.2	78.8 ± 10.7	26.7 ± 4.7	15.8 ± 0.9	4.3 ± 0.4

* Concentration 10 μM

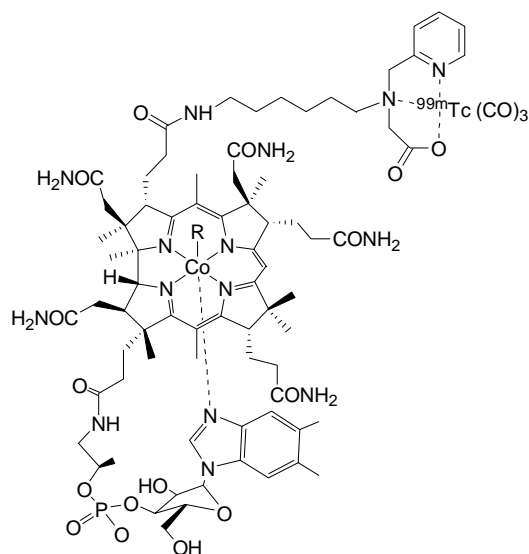
** data presented in Table 1 of the manuscript

Conclusion: The results indicate that a short exposure of the KB cells to antifolates or leucovorin does not significantly influence the tumor cell uptake and internalization of the ^{99m}Tc -PAMA-folate radiotracer. Thus, it can be concluded that α -FR up-regulation in tumor cells unlikely takes place after one hour exposure to antifolates *in vitro* and *in vivo*.

Biodistribution data of ^{99m}Tc -labeled vitamin B₁₂ radiotracer after administration of FA, MTX, PMX and LV

Purpose: Vitamin B₁₂ (= cyanocobalamin) is utilized in intracellular metabolic pathways and therefore an essential cofactor. Many hyperproliferative cells require increased levels of vitamin B₁₂ (Collins *et al.*, Mayo Clinic Proceedings 2000, 75, 568-580). Current research projects in our institute focus on the development of radiolabeled B₁₂-derivatives for radiodiagnostic imaging purposes (van Staveren *et al.*, J Organomet Chem 2004, 689, 4803-4810; van Staveren *et al.*, Helv Chim Acta, 2005, 88, 447-460). The ^{99m}Tc -PAMA(6)-B₁₂ radiotracer (supplemental Fig. 1) was found to significantly accumulate in the renal tissue due to the expression of receptors for transcobalamin II in the kidneys.

In this study we wanted to prove the specificity of the antifolate effect by investigating whether or not antifolates have also an effect on the renal clearance of vitamin radiotracers, which are not re-absorbed via the α -FR.



SUPPLEMENTAL FIGURE 1. Chemical structure of ^{99m}Tc -PAMA(6)-B₁₂ radiotracer.

Experimental Design and Results: Synthesis of the ^{99m}Tc -PAMA(6)-B₁₂ radiotracer. The B₁₂-derivative was functionalized with the PAMA-chelating system via a C6-alkyl-linker system for tridentate coordination of the ^{99m}Tc -core. The radiolabeling of the compound was performed using the IsolinkTM-method and separated from unlabeled ligand by means of HPLC.

Biodistribution studies. Conditions of this *in vivo* study were equal to those reported for the

folate radiotracer (the same mice strain, kept under folate and vitamin B₁₂ deficient rodent chow). FA, LV, MTX or PMX respectively were injected via a lateral tail vein at the indicated time points previous to the administration of the ^{99m}Tc-PAMA(6)-B₁₂ radiotracer. The results are tabulated as percentage of the injected dose per gram (% ID/g) of weight tissue in supplemental Table 4.

SUPPLEMENTAL TABLE 4

Biodistribution 4 h post injection of the ^{99m}Tc-PAMA(6)-B₁₂ radiotracer

	[% ID/g]*				
	Control	Folic acid*	Leucovorin ^a	Methotrexate ^b	Pemetrexed ^b
blood	3.42 ± 0.35	4.91 ± 0.54	4.81 ± 2.72	4.49 ± 0.62	4.35 ± 0.76
heart	3.51 ± 0.35	4.33 ± 0.32	2.87 ± 0.44	4.31 ± 0.45	3.98 ± 0.46
lung	3.87 ± 0.39	5.12 ± 0.46	3.44 ± 0.65	4.75 ± 0.50	4.38 ± 0.80
spleen	3.90 ± 0.21	4.88 ± 0.40	2.94 ± 1.21	5.27 ± 0.48	4.34 ± 1.19
kidney	26.93 ± 12.89	26.98 ± 1.30	26.58 ± 5.99	32.58 ± 5.78	22.04 ± 6.27
stomach	3.20 ± 0.08	4.22 ± 0.54	3.82 ± 1.46	4.39 ± 0.54	4.30 ± 0.99
intestines	4.00 ± 0.40	4.54 ± 0.90	3.35 ± 0.76	4.06 ± 0.81	3.99 ± 0.92
intestinal contents	2.06 ± 0.95	5.28 ± 4.12	3.04 ± 0.22	3.13 ± 0.96	1.34 ± 1.13
liver	10.82 ± 1.04	14.67 ± 0.50	11.08 ± 3.87	14.29 ± 3.75	14.50 ± 2.50
muscle	0.91 ± 0.11	1.12 ± 0.23	0.75 ± 0.05	1.07 ± 0.21	0.94 ± 0.05
bone	1.74 ± 0.32	2.63 ± 0.31	1.64 ± 0.23	2.36 ± 0.16	2.15 ± 0.85

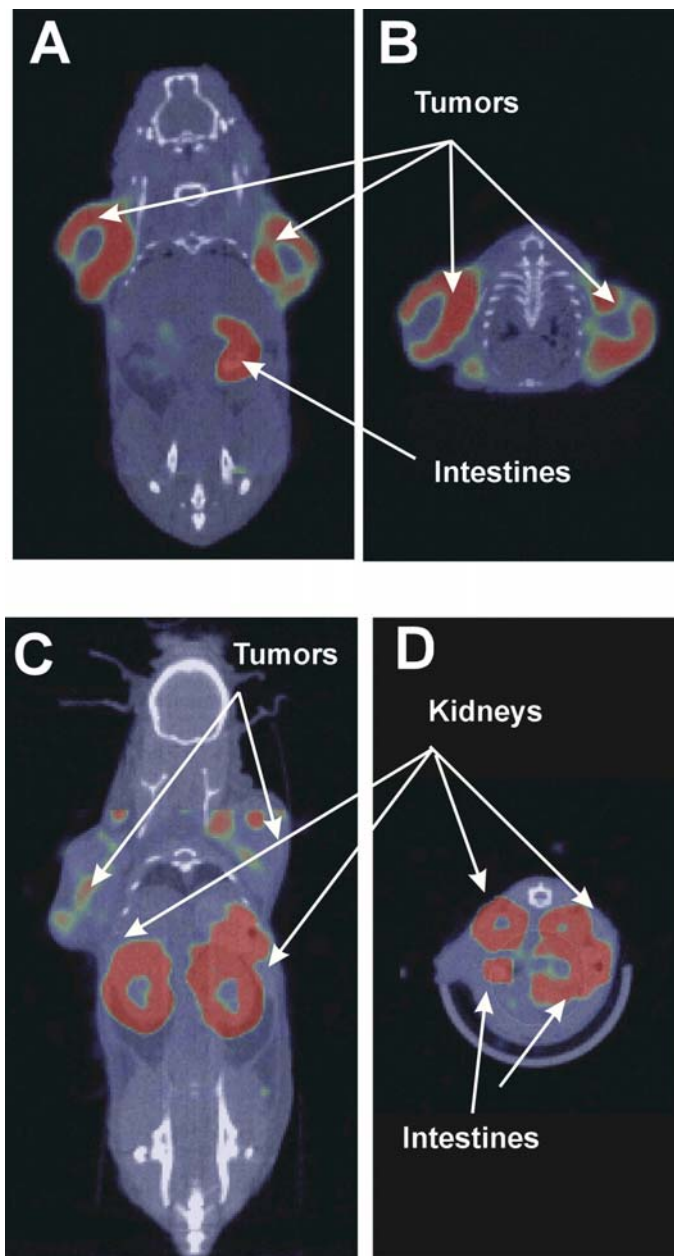
^a co-injected

^b pre-injected 1 h previous to the radiotracer

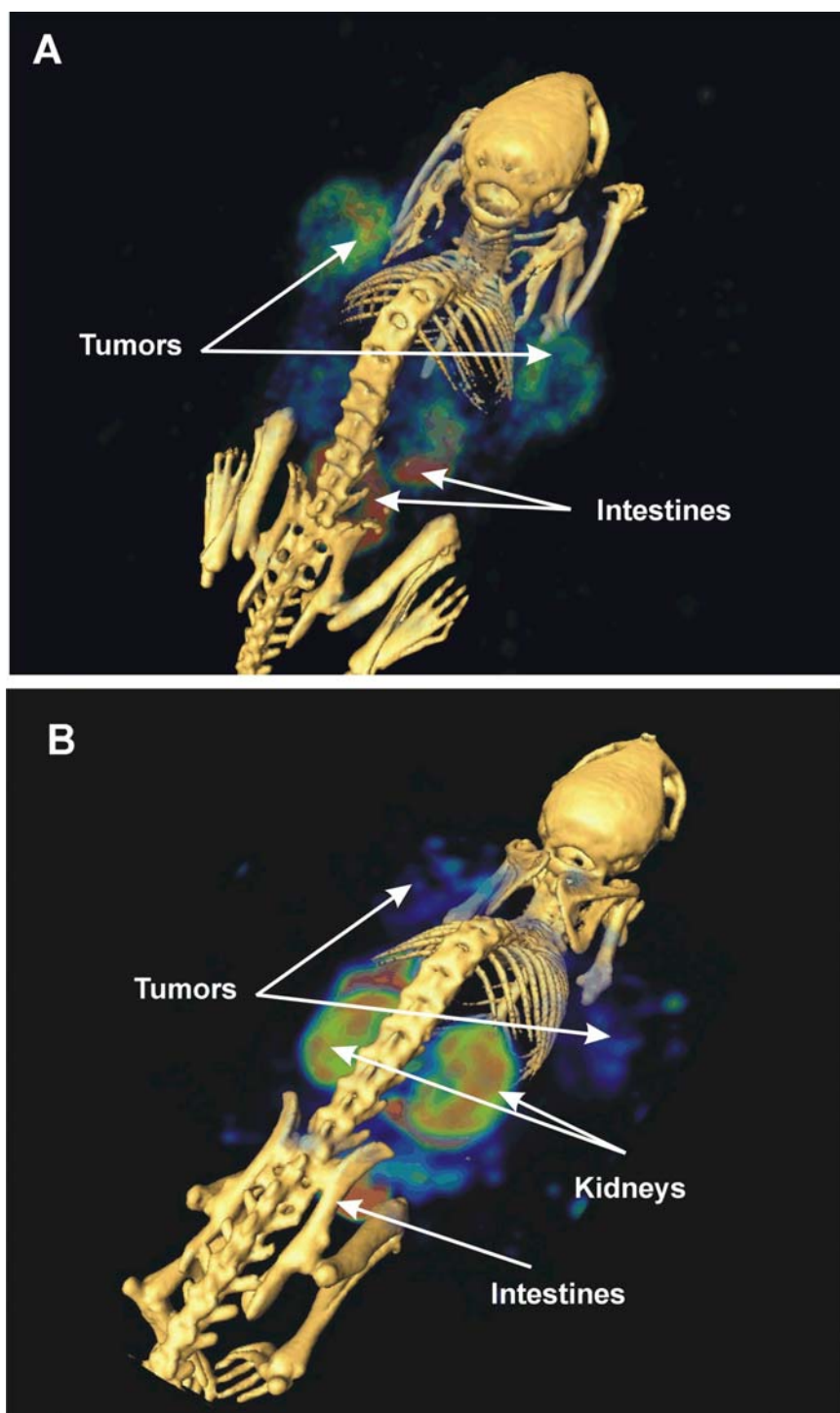
* n = 3

Conclusion: The results clearly provide evidence, that antifolates and LV have no influence on the kidney uptake of ^{99m}Tc-PAMA(6)-B₁₂, because ^{99m}Tc-PAMA(6)-B₁₂ is not reabsorbed in the kidney via α-FR. Hence, the results we observe with ^{99m}Tc-PAMA-folate are specifically related to the affinity of the antifolates or LV to the α-FR.

Supplemental SPECT/CT images



SUPPLEMENTAL FIGURE 2. A&B: Representative, high-resolution coronal and axial SPECT/CT sections of a mouse, pre-injected with PMX before injection of the radiotracer ^{99m}Tc -PAMA-folate. Radioactivity is predominantly accumulated in the outer rim of the tumors. **C&D:** Mouse without PMX pre-injection, but ^{99m}Tc -PAMA-folate only. Radioactivity is predominantly accumulated in the renal cortex. Scans are taken 24 h p.i. of the radiotracer. Scan duration between 30 and 60 min.



SUPPLEMENTAL FIGURE 3. A: High-resolution SPECT/CT of a mouse injected with PMX, 1 h before injection with the radiotracer ^{99m}Tc -PAMA-folate. B: Mouse injected with ^{99m}Tc -PAMA-folate only. Scans are taken 24 h p.i. of the radiotracer. Scan duration between 30 and 60 min.