In vitro and in vivo targeting of different folate receptor-positive cancer cell lines with a novel ^{99m}Tc-radiofolate tracer

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Abstract. *Purpose:* For the assessment of folate-based radiopharmaceuticals, human nasopharyngeal KB carcinoma cells are traditionally used although nasopharyngeal cancer is rare. On the other hand, the folate receptor (FR) is frequently overexpressed on diverse cancer types, the highest frequency (>90%) being on ovarian carcinomas. The goal of our study was the in vitro and in vivo assessment of different FR-positive human carcinoma cells. In addition, a murine sarcoma cell line was assessed as a pre-clinical alternative to human xenograft models.
Methods: FR-positive human nasopharyngeal, cervical, ovarian and colorectal cancer cell lines and the transgenic mouse sarcoma (24JK-FBP) cell line were targeted with a novel ^{99m}Tc-tricarbonyl folate derivative **2**. Comparative in vitro cell binding studies were carried out under standardised folate-deficient conditions. In vivo studies were performed in nude mice and C6 black mice.
Results: The in vitro cell experiments revealed only FR-specific binding (unspecific <0.02%), ranging from 3.5% to 52% of complex **2** owing to variable levels of FR expression of the cell lines. In vivo tumour uptake of radiotracer **2** varied less than in vitro. It ranged from 0.66±0.17% ID/g (LoVo) through 1.16±0.64% ID/g (IGROV-1) and 1.55±0.43% ID/g (24JK-FBP) to 2.33±0.36% ID/g (KB) 4 h p.i.
Conclusion: These pre-clinical studies indicate that in vitro data obtained in FR-positive cancer cells do not necessarily correspond with or predict in vivo radiofolate uptake in corresponding (xeno)grafts. In addition, the murine 24JK-FBP cell line proved to be a valuable pre-clinical alternative to human tumour models.

Keywords: Folates – Tumour targeting – Pre-clinical studies – Diagnostic potential

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Introduction

About 15 years ago, an ovarian cancer-associated, highly overexpressed antigen (CaMOv18) was identified as a high-affinity folate-binding protein. This antigen was later recognised as the folate receptor (FR). The frequent overexpression of the FR on malignant tissue provides a unique opportunity to specifically target cancer cells by virtue of its high affinity for folic acid and folate analogues. FR targeting has been exemplified using folate conjugates with a wide variety of diagnostic and therapeutic probes [1–3] (e.g. chemotherapeutic agents [4], protein toxins [5, 6], antisense oligonucleotides [7, 8], MRI contrast agents [9] and liposomes [10–12]). Folate radiopharmaceuticals have been developed as imaging agents of FR-positive cancers, whereof an ^{111}In- and a ^{99m}Tc-radiolabelled derivative have already proceeded into clinical trials [13–17]. This highlights the potential of the FR for tumour targeting and encouraged us to develop and evaluate a novel organometallic ^{99m}Tc-folate radiopharmaceutical for diagnosis of FR-positive tumours [18].

The human nasopharyngeal KB carcinoma cell line can be considered the "gold standard" for FR-targeting studies. KB cells are traditionally used because of their high and constant FR expression level [13, 14, 19–24]. However, the number of patients suffering from nasopharyngeal cancer is relatively small. In North America, nasopharyngeal cancer is responsible for only about 0.25% of all cancer types, which means approximately 1,000 cases per year according to the American Cancer Society. However, the FR is overexpressed in a number of other epithelial carcinomas (e.g. ovarian, endometrial, renal, breast, lung, colorectal), with ovarian cancer revealing the highest frequency (>90% of cases) [1, 25]. At the same time, ovarian cancer is the seventh most common cancer (other than skin cancer) in women, ranking fourth as the cause of cancer death in women. The poor prognosis in ovarian cancer is basically

due to the advanced stage of the disease at the time of a possible diagnosis. Thus, methods for precise, early and non-invasive detection of ovarian cancer are of crucial interest. In this respect, folate radiopharmaceuticals have a prominent role.

The goals of the present work were twofold: First, we wished to assess different FR-positive human cancer cell lines, namely ovarian (IGROV-1, SKOV-3), cervical (HeLa) and colorectal (HT-29, LoVo) carcinomas. These data should be compared with those obtained with the most often used nasopharyngeal KB cancer cell line [4, 25–28]. Secondly, we aimed to evaluate a murine sarcoma cell line (24JK-FBP [29, 30]), transfected with the human FR-α gene, for its potential to serve as an inexpensive (with respect to the mouse strain) alternative to human xenograft models. For these experiments we employed a novel organometallic $^{99m}Tc(CO)_3$-folate radiotracer **2** which proved to target FRs of different cancer cell lines in vitro and in vivo [31].

Materials and methods

General

Picolylamine mono acetic acid (PAMA)-γ-folate **1** was synthesised as previously reported [18]. [3′,5′,7,9-^{3}H]Folic acid potassium salt (37 MBq/ml, 888 GBq/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). The scintillation solution Ultima Gold, high flash-point LSC cocktail was purchased from Packard Company (Groningen, the Netherlands). Precursor $[^{99m}Tc(CO)_3(OH_2)_3]^+$ was prepared using the Isolink-kit (Mallinckrodt-Tyco, Petten, the Netherlands). [Na][$^{99m}TcO_4$] was eluted from a $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt-Tyco, Petten, the Netherlands) with a 0.9% saline solution. KB (CCL-17) and A375 cells (CRL-1619) were purchased from ATCC (American Type Culture Collection, Manassas, USA). HeLa (ACC 57), KB-V1 (ACC 149), LoVo (ACC 350) and HT-29 (ACC 299) were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig Germany). The ovarian cancer cell lines IGROV-1 and SKOV-3 were kindly provided by Dr. Silvia Miotti (Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy) and Dr. Ellen Vitetta (University of Texas, Dallas, USA), respectively. The 24JK-FBP cells were obtained from Dr. Patrick Hwu (National Cancer Insitute, Bethesda, MD, USA). Special RPMI cell culture medium (without folic acid, vitamin B_{12}, phenol red) was purchased from Cell Culture Technologies GmbH, Gravesano/Lugano, Switzerland. HPLC analyses were performed on a Merck-Hitachi L-6200A-system, equipped with an L-3000 tunable absorption detector, a Berthold LB 508 radiometric detector and an XTerra (Waters) MS C-18 reversed phase column (5 µm, 15 cm×4.6 mm). HPLC solvents: aqueous 0.05 *M* triethylammonium phosphate buffer, pH 7.0 (solvent A), methanol (solvent B). The HPLC system started with 100% A with a linear gradient to 20% A and 80% B over 15 min, followed by 5 min of 100% A with a flow rate of 1 ml/min. Radioactivity (γ-radiation of ^{99m}Tc) was measured with a γ-counter (Cobra II, Model B 5003, Packard) and (β-radiation of ^{3}H) with a β-counter (TRI-CARB, 1900 TR, Liquid Scintillation Analyzer, Packard). Protein concentrations for the in vitro experiments were measured with a microplate reader (Bio-Rad, Model 550), using a Micro BCA Protein Assay kit (Prod # 23235), Socochim.

Description of the cancer cell lines

IGROV-1 and SKOV-3 cells The IGROV-1 and SKOV-3 cell lines are human ovarian adenocarcinoma cell lines. It has been reported that IGROV-1 cells express higher levels of FRs than SKOV-3 [28]. Ovarian cancer has been shown to overexpress the FR with the highest frequency (>90%) amongst the FR-positive tumour types [1, 25, 32]. For these reasons, ovarian cancers are of primary concern for FR-targeting strategies, with regard not only to diagnosis but also to potential therapy with folate radiopharmaceuticals. FR expression in normal and malignant tissue of the ovary, cervix and uterus was investigated in detail by Wu et al. [33]. The results of their studies showed that these tissues present different patterns of FR regulation in differentiation and malignancy. Quantitatively increased FR expression associated with foci of poor differentiation could be shown within an FR-expressing ovarian tumour. In agreement with these findings, Toffoli et al. found a positive correlation between FR expression and advanced stage of ovarian tumours [34].

LoVo and HT-29 cells LoVo and HT-29 cell lines are colon adenocarcinoma cells. Campbell et al. found high levels of FR expression in LoVo cells, but only low FR expression in HT-29 cells [26]. It has also been reported that the HT-29 cell line is able to adapt to the culture conditions by overexpression of FRs [35]. Garin-Chesa et al. demonstrated that frequency of FR expression is lower in colorectal carcinoma tissues than in ovarian cancer types [25].

KB, KB-V1 and HeLa cells KB cells are most often reported in the literature as a valuable test system for in vitro and in vivo studies of FR-targeting agents [14, 21, 22, 29, 36, 37]. This cell line is usually referred to as a human nasopharyngeal carcinoma cell line. The nasopharynx is an area at the back of the nose towards the base of the skull. Cancers that develop in this area tend to spread widely and can hardly be treated by surgery. However, nasopharyngeal cancer is relatively rare, accounting for only about 0.25% of all cancers in North America. The KB-V1 cell line is a multidrug-resistant (mdr) subclone derived from KB-31 cells in 1985. KB-V1 cells strongly express mdr1 mRNA and protein. Official cell banks concordantly report that KB cells have to be considered as a HeLa cell subclone according to the DNA fingerprint. HeLa cells were established from an epitheloid cervical carcinoma. Cervical cancer begins in the lining of the cervix, the lower part of the uterus. The development of cervical cancer often takes a number of years, but can sometimes happen more quickly. The American Cancer Society predicts that there will be about 10,000 new cases of invasive cervical cancer in the United States in 2005.

24JK-FBP cells This is a mouse sarcoma cell line which was retrovirally transfected with the human FR-α gene. The FR expression level of this cell line resembles closely that of human ovarian cancer cells [29].

A375 cells The human A375 cell line is a malignant melanoma cell line. It has been reported in the literature that this cell line can be used as a reliable negative control for FR-targeting studies because it lacks FRs [4].

Cell cultures

Human and murine cancer cell lines were cultured continuously in a 150-cm^2 flask as monolayers at 37°C in a humidified atmosphere containing 7.5% CO_2. The cells were cultured in FFRPMI medium (modified RPMI 1640 medium without folic acid, vitamin B_{12} and phenol red), supplemented with 10% heat-inactivated fetal calf serum (FCS, as the only source of folate), L-glutamine and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, fungizone 0.25 µg/ml). Cell culture media such as FCS-supplemented

Table 1. B_{max} and K_D values of ^{3}H-folic acid in different FR-positive cell lines

Cell line	B_{max} (pmol/mg protein)	K_D (n*M*)
KB cells[a]	11.9 (−12.18 to +35.97)	7.22 (−11.15 to +25.58)
IGROV-1 cells[a]	0.09 (−1.11 to +0.28)	3.45 (−28.70 to +35.60)
24JK-FBP cells[a]	0.16 (+0.09 to +0.22)	1.24 (−2.95 to +5.43)

[a]Values shown in parentheses represent the lower and upper limits of the 95% confidence interval; number of experiments =2

FFRPMI are known to feature a final folate concentration of ~3 n*M*, i.e. a value at the low end of the physiological concentration in human serum [38]. Cell preparation for in vitro experiments was as follows: 18–20 h prior to each experiment, the cells were seeded in 12-well plates (7–8×10^5 cells/well) and incubated at 37°C to form confluent monolayers overnight. Experiments were performed in triplicate. Cell preparation for in vivo experiments was as follows: For subcutaneous inoculation of the mice, subconfluent cells were harvested by treatment with EDTA (1 m*M*) in phosphate-buffered saline (PBS, 1x, pH 7.4). The cells were then washed once with PBS and pelleted by spinning at 1,000×*g* for 5 min at 20°C. The cells were resuspended in PBS to the corresponding concentrations (35–50×10^6 cells/ml).

Determination of B_{max} and K_D values of KB, IGROV-1 and 24JK-FBP cells

Inhibition experiments to determine binding constants (K_D, B_{max}) were performed according to the following procedure: Cell monolayers were washed with pure ice-cold PBS, followed by addition of FFRPMI medium (475 µl, without FCS/L-glutamine/antibiotics) in each well. 500 µl of the corresponding ice-cold folic acid solution (10 concentrations ranging from 0.001 µ*M* up to 0.2 µ*M*, prepared in PBS, pH 7.4) was added. The well plates were pre-incubated at 4°C for 40 min. The solution of ^{3}H-folic acid (25 µl, ~19 kBq) was added and the well plates incubated again at 4°C for 2 h[1]. Cells were then washed three times with PBS (pH 7.4, 1 ml) and the cells were lysed in 1 *N* NaOH (1 ml), transferred to 4-ml tubes and homogenised by vortex. The concentration of proteins was determined in each sample by a Micro BCA Protein Assay kit. Scintillation solution (4 ml) was added, and the samples were homogenised and transferred into scintillation flasks. Each sample was counted for radioactivity in a β-counter. K_D and B_{max} values had been determined by converting the raw data from counts per minute (cpm) bound per mg protein into pmol radiotracer bound per mg protein, and analysed by Rosenthal plot. The mean values of K_D and B_{max} acquired in two independent experiments for each cell line are shown in Table 1.

Preparation of the radiotracer **2** *and solutions for in vitro and in vivo experiments*

^{99m}Tc(CO)$_3$-PAMA-γ-folate (**2**) was synthesised according to the following procedure: Precursor *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ was prepared via the Isolink method [18, 39]. The obtained solution of *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ (100 µl; ~1 GBq/ml), PBS (pH 7.4, 350 µl) and a stock solution of derivative 1 (50 µl, 10^{-3} *M* in PBS) were mixed in a sealed glass vial. The reaction was heated at 75°C for 30 min to form complex **2** (yield >95%, Fig. 1). Complex **2** was separated from unlabelled compound **1** by means of HPLC and diluted in PBS (pH 7.4) to obtain a final concentration of 1 MBq/ml for in vitro experiments or 3.7 MBq/ml for in vivo experiments. The complex **2** revealed a high stability in PBS and human plasma over a period of 24 h at 37°C, as previously demonstrated by our group [18].

Cell uptake in vitro

Cell binding experiments with radiotracer **2** were performed according to the following procedure: Cell monolayers were rinsed twice with ice-cold PBS (pH 7.4). Pure FFRPMI medium (975 µl, without FCS/L-glutamine/antibiotics) or 475 µl FFRPMI and 500 µl of a solution of folic acid (200 µ*M*) were added into each well. The well plates were pre-incubated at 37°C for 40 min. Then, a solution of the ^{99m}Tc(CO)$_3$-labelled radiotracer (25 µl, 1 MBq/ml) was added and the well plates were incubated again at 37°C for 1 h. The cell monolayers were washed with PBS for determination of total bound and internalised radiotracers or with stripping buffer (aqueous solution of 0.1 *M* acetic acid and 0.15 *M* NaCl, pH 3) in order to

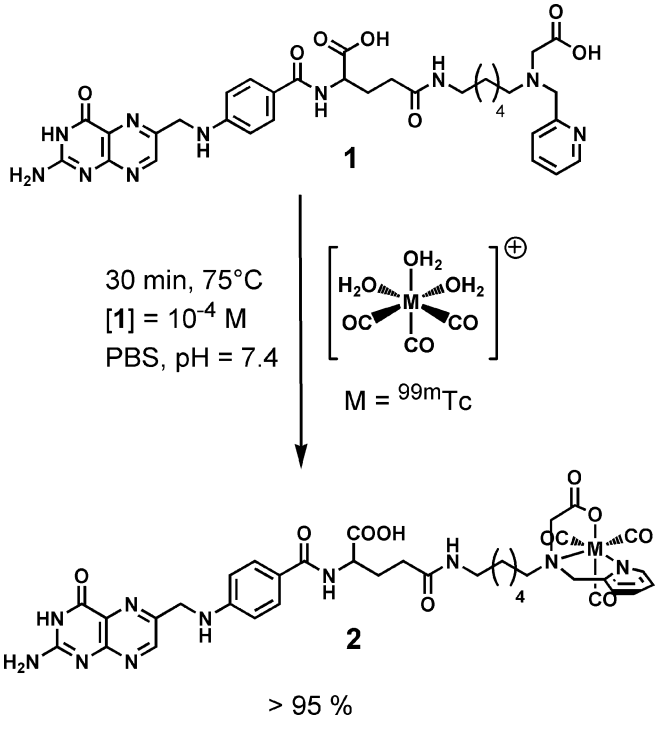

Fig. 1. Preparation of new, organometallic ^{99m}Tc(CO)$_3$-folate radiotracer **2**

[1]Experiments were performed with ^{3}H-folic acid at 4°C because under these conditions endocytosis is largely attenuated. Time-dependent cell binding studies, performed over a period of 4 h revealed maximal binding after 2 h. Experiments with excess unlabelled folic acid in order to block FRs revealed negligible amounts (<0.5%) of unspecific cell binding of ^{3}H-folic acid and could therefore be neglected (data not shown).

determine exclusively the internalised fraction. Blocking experiments were performed by pre-incubation of the cells with excess folic acid (100 μ*M*) in order to saturate FRs. The monolayers were dissolved in 1 *N* NaOH (1 ml), transferred to 4-ml tubes and homogenised by vortex. The samples were counted for radioactivity using a γ-counter. The concentration of proteins was determined for each sample by a Micro BCA Protein Assay kit in order to standardise measured radioactivity in each sample to the averaged content of proteins in each well. The raw data of measured radioactivity were converted into percentage of total added radioactivity.

In vivo studies

Four- to 5-week-old female, athymic nude mice (CD1-Foxn1/*nu*) and female black mice (C57BL/6N) were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were housed under conditions of controlled temperature (26°C and 22°C, respectively), humidity (68%) and daily light cycle (12 h light/12 h dark). The animals were exclusively fed with a folate-deficient rodent chow (in order to reduce their serum folate to a level near that of human serum) [36]. After an acclimation period of 5–7 days, the mice were inoculated subcutaneously with 0.1 ml tumour cell suspension ($35–50\times10^6$ cells/ml) into the subcutis of the axilla. Radiotracer distribution studies were performed 12–14 (or 19–21 days in the case of IGROV-1 cells) after tumour cell inoculation. Biodistribution studies were performed in triplicate. The radiotracer **2** (100 μl, 3.7 MBq/ml) was administered via a lateral tail vein. To competitively block tissue FRs, mice were injected with excess folic acid (0.25 μmol in PBS, pH 7.4) 5 min prior to the radiotracer. At the indicated points in time the animals were sacrificed and dissected. The selected tissues were removed and weighed, and the radioactivity was counted by a γ-counter to determine tissue distribution of the radiotracers within the test animal. The results were tabulated as percentage of the injected dose per gram tissue (% ID/g), using reference counts from a definite sample of the original injectate.

Results

In vitro studies

The aim of these screening experiments was the qualitative evaluation of the in vitro uptake of the ^{99m}Tc-folate radiotracer **2** (Fig. 1) in different FR-expressing cancer cell lines (for detailed description of the cell lines, see Materials and methods). A series of FR-positive and FR-negative human and murine cancer cell lines were tested. Results of cell binding and internalisation experiments are summarised in Fig. 2. Radioactivity bound to FR-positive KB and the multiresistant subclone KB-V1 cell lines was found in the range of 35–50% of total added radioactivity. Among the human FR-positive ovarian carcinoma cell lines, we found slightly higher cell binding in IGROV-1 cells (~42%) than in SKOV-3 cells (~26%). Also, for the human cervical HeLa carcinoma cell line (~36%) and the transfected murine sarcoma cell line (~36%) we found results in the same range. Colorectal carcinoma cell lines (LoVo and HT-29) revealed relatively low binding (3–5% of total added radioactivity). As expected, the FR-negative A375 cell line was found to bind less than 0.05% of total added radioactivity. The internalised fraction of the radiotracer was determined by washing the cells with stripping buffer (pH 3), enabling the acid-dependent release of FR-bound radiotracer on the cell surface. For all FR-positive cell lines, internalisation amounted to approximately 20–35% of total cell-associated radioactivity. Blockade of the receptors with excess folic acid resulted in less than 0.1% binding of the radiotracer **2** in each of the FR-positive cell lines. It is noteworthy that the FR-negative A375 could be readily cultured in folic acid-containing standard medium. On the other hand, these cells survived for only a short time in folate-deficient medium in which FR-positive cancer cell lines could be easily cultured.

In vivo studies

For the in vivo assessment of all FR-positive tumour cell lines, we performed biodistribution studies in tumour-bearing mice, 4 h after administration of the radiotracer **2**. Previous in vivo studies in KB tumour-bearing mice have shown the most favourable tumour accumulation of the radiotracer at this time after injection.

Compound **2** predominately accumulated in FR-positive tissues (tumour and kidneys). The amounts of radioactivity found in non-targeted tissue and organs were in the same range for all experiments performed with athymic nude mice (CD1-Foxn1/*nu*) as well as with C6 black mice (C57BL/6N). Representatively, the % ID/g data of radioactivity accumulated in tumours and kidneys as well as the tumour-to-kidney, tumour-to-blood and tumour-to-liver ratios are presented in Table 2. Uptake of radioactivity in KB-V1 tumours (1.48±0.23% ID/g) was lower than in KB tumours (2.33±0.36% ID/g) and tumour-to-blood ratios of accumulated radioactivity were also slightly different (KB-V1: 26.9±18.9 vs KB: 58.0±12.2). However, tumour-to-kidney ratios of radioactivity were comparable (KB-V1: 0.12±0.03 vs KB: 0.13±0.02). Data obtained from HeLa cell tumour-bearing mice (1.31±

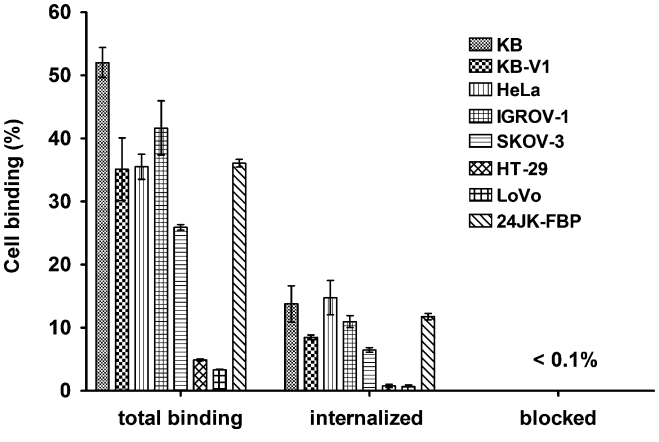

Fig. 2. Cell binding, internalisation and competitive inhibition (with 100 μ*M* folic acid) after incubation (1 h at 37°C) of the radiotracer **2**, performed with different FR-positive human and murine cancer cell lines

0.06% ID/g) were similar to those of KB-V1 cell tumours, but lower than in KB tumours, and tumour-to-blood ratios (21.10±4.60) and tumour-to-kidney (0.08±0.02) ratios were accordingly reduced. Differences in accumulated radioactivity found in vitro between the two ovarian (IGROV-1, SKOV-3) cell lines were less pronounced in vivo. Tumour uptake of the radiotracer in mice with IGROV-1 (1.16±0.64% ID/g) and SKOV-3 (1.03±0.18% ID/g) cell xenografts was found to be in the same range as in animals with HeLa cell tumours. Tumour-to-blood ratios were also found to be in the same range (IGROV-1: 23.11± 16.33; SKOV-3: 19.97±5.59). On the other hand, we observed very low in vivo tumour uptake of the radiotracer in colorectal LoVo and HT-29 cancers (~0.6% ID/g), leading to unfavourably low tumour-to-non-tumour ratios (tumour-to-blood ratios: 7.69±1.66 and 6.53±1.12; tumour-to-liver ratios: 0.43±0.15 and 0.30±0.17; tumour-to-kidney ratios: 0.04±0.01 and 0.06±0.03, respectively). Murine 24JK-FBP sarcoma cells, studied in normal C6 black mice, revealed highest tumour accumulation of radioactivity (1.55±0.43% ID/g) amongst all cell lines tested (except KB cells), and tumour-to-background ratios were comparable to those obtained in mice bearing nasopharyngeal (KB-V1), ovarian (IGROV-1, SKOV-3) or cervical (HeLa) tumour xenografts.

More detailed, time-dependent (1 h, 4 h and 24 h p.i.) in vivo studies were performed in the two human FR-positive cell lines KB, IGROV-1 and the murine cell line 24JK-FBP in athymic nude mice (KB, IGROV-1) and C6 black mice (24JK-FBP) (Tables 3, 4 and 5). As mentioned before, for pre-clinical studies a mouse cell line is of high interest for two reasons: (a) it represents an inexpensive tumour model because it enables in vivo studies using normal mice (C57BL/6N) rather than the costly athymic nude mice necessary for experiments with human tumour xenografts (e.g. KB); (b) particularly for 24JK-FBP cells, it has been reported in the literature that their FR level mimics very well that found in clinically relevant human ovarian cancer cell lines (e.g. IGROV-1) [29].

Tumour uptake 4 h p.i. of the radiotracer was slightly lower in IGROV-1 and 24JK-FBP tumours than in KB cell tumours. Interestingly, accumulation of radioactivity in KB tumours was highest 4 h p.i. of the radiotracer (2.33±0.36% ID/g), whereas maximal tumour uptake of radiotracer was found 1 h p.i. in IGROV-1 (1.94±0.20% ID/g) and 24JK-FBP cell tumours (1.90±0.09% ID/g). The radiotracer was efficiently cleared from the bloodstream, resulting in <0.15% ID/g 4 h after injection of radioactivity. Tumour-to-blood and tumour-to-liver radioactivity ratios increased over time and reached maximum values after 24 h (tumour-to-blood ratio: approx. 100–300 and tumour-to-liver ratio: approx. 6–25). Tumour-to-kidney ratios were virtually constant (within the standard deviations) over time for all types of (xeno)graft. Accumulation of the radiotracer **2** was reduced to background levels (<0.01% ID/g) in all three tumour types when excess free folic acid was intravenously injected 5 min prior to the radiotracer in order to saturate FRs. These last findings clearly proved that FR-specific tumour accumulation of the radiotracer also occurs in vivo.

Correlation of tumour size and radiotracer uptake

Tumour size of the KB, IGROV-1 and 24JK-FBP cell (xeno)grafts varied, even though the number of inoculated tumour cells and the period of tumour growth were standardised. Different tumour size/mass can potentially give rise to artefacts in measured % ID/g values because of reduced accumulation of radiotracer if cells buried deep within large tumours become poorly accessible to blood-borne radiotracers. Therefore, we performed studies in order to correlated % ID/g values of KB (fast-), 24JK-FBP (medium-) and IGROV-1 (slow-growing) tumours with the corresponding tumour sizes. KB tumours grew faster in vivo than did 24JK-FBP, whereas only slow tumour growth was observed with IGROV-1 cells, which grew no bigger than approximately 150 mg even 3 weeks after inoculation of the tumour cells. Tumour data were obtained from animals 2 and 3 weeks after inoculation of the cancer cells. Radioactivity was determined 4 h p.i. of the radiotracer **2**.

Figure 3 depicts the percentage injected dose (% ID) plotted against the corresponding tumour size for each type of (xeno)graft (30–300 mg). Good linear correlation (r>0.97) between accumulated radiotracer **2** and the tumour size was found in all tumour types. Hence, one can conclude that small variations in the tumour size did

Table 2. Biodistribution of $^{99m}Tc(CO)_3$-PAMA-γ-folate (**2**) in athymic nude mice bearing human cancer cell xenografts 4 h post injection

Cell line	Tumour	Kidney	Tumour-to-kidney ratio	Tumour-to-blood ratio	Tumour-to-liver ratio
KB	2.33±0.36	18.48±0.72	0.13±0.02	58.0±12.2	2.53±2.13
KB-V1	1.48±0.23	12.05±0.98	0.12±0.03	26.91±18.92	4.79±3.63
HeLa	1.31±0.06	16.07±3.47	0.08±0.02	21.10±4.60	0.78±0.09
IGROV-1	1.16±0.64	12.75±3.42	0.09±0.03	23.11±16.33	0.99±0.22
SKOV-3	1.03±0.18	8.00±1.70	0.13±0.02	19.97±5.59	0.71±0.26
LoVo	0.66±0.17	16.25±4.11	0.04±0.01	7.69±1.66	0.43±0.15
HT-29	0.67±0.03	12.43±4.80	0.06±0.03	6.53±1.12	0.30±0.17
24JK-FBP[a]	1.55±0.43	17.13±1.50	0.09±0.03	21.07±12.36	4.88±2.25

Values (% ID/g) represent the mean±SD of data from three animals per cohort

[a]Mouse sarcoma grafts in female black mice (C57BL/6N)

Table 3. Time-dependent biodistribution of $^{99m}Tc(CO)_3$-PAMA-γ-folate (**2**) in KB tumour-bearing mice

	$^{99m}Tc(CO)_3$-PAMA-γ-folate (**2**)			+ Folic acid[a]
	1 h	4 h	24 h	4 h
Blood	0.09±0.02	0.04±0.00	0.01±0.00	0.01±0.00
Heart	0.85±0.27	0.32±0.07	0.02±0.02	0.00±0.00
Lung	0.57±0.19	0.33±0.02	0.03±0.00	0.02±0.02
Spleen	0.20±0.03	0.15±0.04	0.01±0.02	0.01±0.01
Kidney	14.37±1.35	18.48±0.72	6.90±0.72	0.06±0.01
Stomach	0.31±0.06	0.63±0.33	3.70±6.37	3.80±5.46
Intestines	1.89±0.37	1.49±0.23	0.76±1.15	0.40±0.45
Liver	0.89±0.31	2.37±2.85	0.10±0.02	0.12±0.02
Muscle	1.06±0.33	0.54±0.05	0.05±0.04	0.04±0.01
Bone	0.76±0.22	0.31±0.07	0.03±0.04	0.01±0.01
Tumour	1.30±0.61	2.33±0.36	1.32±0.17	0.00±0.01
Tumour-to-blood ratio	15.82±11.29	58.0±12.2	255.4±115.4	
Tumour-to-liver ratio	1.60±0.81	2.53±2.13	13.77±1.90	
Tumour-to-kidney ratio	0.09±0.05	0.13±0.02	0.19±0.04	

Values (% ID/g) represent the mean±SD of data from three animals per cohort
[a]0.25 μmol; injection 5 min before radiotracer **2**

not influence our results with respect to accumulated radioactivity, and therefore the results obtained with each of these tumour cell lines can be compared even if the tumour masses did not match throughout the experiments.

Discussion

The potential of the novel radiofolate **2** to target KB carcinoma cells in vitro and in vivo with high FR specificity was demonstrated previously. Because, overall, the data were comparable with those reported for other radiofolates (e.g. ^{111}In-DTPA-folate [13], ^{99m}Tc-DTPA-folate [14]), it is reasonable to assume that the results reported in the present study are largely transferable to other (radio)folate derivatives. The experiments using the radiotracer **2** with various cancer cell lines led to several interesting observations.

Qualitative in vitro studies showed specific cell binding of the folate radiotracer **2** in all FR-positive human and murine cancer cell lines. This was proven in blocking experiments with excess folic acid for FR saturation, which

Table 4. Time-dependent biodistribution data of $^{99m}Tc(CO)_3$-PAMA-γ-radiotracer (**2**) in IGROV-1 tumour-bearing mice

	$^{99m}Tc(CO)_3$-PAMA-γ-folate (**2**)			+ Folic acid[a]
	1 h	4 h	24 h	4 h
Blood	0.11±0.05	0.06±0.04	0.00±0.00	0.01±0.02
Heart	0.59±0.08	0.20±0.04	0.01±0.00	0.00±0.00
Lung	0.53±0.05	0.35±0.16	0.02±0.00	0.01±0.00
Spleen	0.12±0.05	0.12±0.08	0.00±0.00	0.04±0.06
Kidney	16.97±3.07	12.75±3.42	4.68±0.13	0.09±0.04
Stomach	0.70±0.11	0.42±0.20	0.21±0.24	22.93±23.10
Intestines	8.78±4.23	1.38±0.71	0.04±0.02	0.71±0.75
Liver	4.61±1.51	1.34±1.07	0.02±0.00	0.40±0.45
Muscle	0.72±0.07	0.37±0.11	0.01±0.00	0.00±0.00
Bone	0.31±0.07	0.15±0.03	0.01±0.02	0.00±0.00
Tumour	1.94±0.20	1.16±0.64	0.66±0.06	0.00±0.00
Tumour-to-blood ratio	25.10±10.74	23.11±16.33	102.3±95.8	
Tumour-to-liver ratio	0.44±0.03	0.99±0.22	27.00±2.34	
Tumour-to-kidney ratio	0.13±0.02	0.09±0.03	0.14±0.00	

Values (% ID/g) represent the mean±SD of data from three animals per cohort
[a]0.25 μmol; injection 5 min before radiotracer **2**

Table 5. Time-dependent biodistribution data of $^{99m}Tc(CO)_3$-PAMA-γ-radiotracer (**2**) in 24JK-FBP tumour-bearing mice

	$^{99m}Tc(CO)_3$-PAMA-γ-folate (**2**)			+ Folic acid[a]
	1 h	4 h	24 h	4 h
Blood	1.77±2.84	0.11±0.09	0.02±0.02	0.00±0.00
Heart	0.72±0.13	0.15±0.05	0.01±0.02	0.00±0.00
Lung	1.27±0.55	0.35±0.04	0.03±0.01	0.00±0.00
Spleen	0.33±0.27	0.08±0.03	0.01±0.01	0.00±0.00
Kidney	15.25±1.00	17.13±1.50	10.92±3.05	0.08±0.01
Stomach	2.72±0.89	0.73±0.33	0.41±0.48	0.07±0.09
Intestines	24.38±13.82	2.77±1.15	1.52±1.37	0.03±0.04
Liver	6.35±3.17	0.35±0.10	0.12±0.06	0.19±0.26
Muscle	1.03±0.13	0.32±0.03	0.05±0.04	0.06±0.01
Bone	0.76±0.11	0.11±0.04	0.01±0.01	0.00±0.00
Tumour	1.90±0.09	1.55±0.43	0.57±0.04	0.00±0.00
Tumour-to-blood ratio	9.62±8.31	21.07±12.36	97.16±114.46	
Tumour-to-liver ratio	0.52±0.20	4.88±2.25	6.01±3.74	
Tumour-to-kidney ratio	0.13±0.01	0.09±0.03	0.06±0.02	

Values (% ID/g) represent the mean±SD of data from three animals per cohort
[a]0.25 μmol; injection 5 min before radiotracer **2**

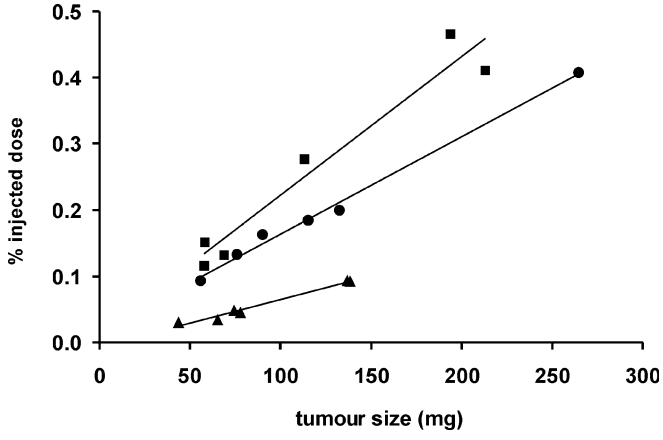

Fig. 3. Correlation of tumour-accumulated radioactivity (% ID) and tumour size (mg): ■ KB tumours (r=0.971), ● 24JK-FBP tumours (r=0.997), ▲ IGROV-1 tumours (r=0.989)

resulted in almost complete inhibition of radiotracer accumulation in these cancer cell lines. FR specificity was further corroborated by the observation that FR-negative cancer cells (A375) were not capable of accumulating the folate radiotracer **2**. However, the fact that cell-associated radioactivity in vitro varied significantly amongst the tested cell lines strongly indicates that the cell lines reveal different FR expression levels. Therefore determination of FR expression level (using ^{3}H-folic acid) was carried out in three representative cell lines (KB, IGROV-1 and 24JK-FBP). It could be shown that KB cells exhibited a distinctively higher level of binding sites (approx. two orders of magnitude) than IGROV-1 or 24JK-FBP cell lines (Table 1).

For the above-mentioned comparative in vitro experiments, all cell lines were cultured under the same standardised conditions using a folate-deficient cell culture medium. However, apart from extracellular folate concentration, ingredients of the cell culture medium and duration of folate-starved conditions (all aspects taken into account in these experiments), genetic properties of the cell lines themselves can influence FR expression levels and, as a consequence, radiotracer uptake. Variable FR expression levels in different cancer cell lines are also reported in the literature. Campbell et al. found (via fluorescence-activated cell sorting) similar FR expression levels in SKOV-3 and HT-29, higher levels in LoVo cells and very high levels in HeLa cells [26], whereas our data clearly indicated significantly higher levels in the ovarian (IGROV-1, SKOV-3) and cervical (HeLa) cancer cell lines than in colorectal cancers (Lovo, HT-29). In order to quantitatively determine FRs in cells and tissues, it is reasonable to use ^{3}H-folic acid since this has many advantages over other methods (e.g. antibodies [25]), in particular high sensitivity thanks to the extremely specific binding of ^{3}H-folic acid to the FR and the fact that only "functional" FRs will be targeted [32].

It is noteworthy that FR-expressing cell lines exhibited an unambiguous advantage over FR-negative cells, such as A375, with regard to proliferation under folate-deprived conditions: the latter cells could hardly be cultured in folate-deficient medium. This means that under folate-starved culture conditions, other folate transport mechanisms (e.g. the ubiquitously distributed reduced folate carrier) are not sufficient for the accumulation of folates, necessary for cell survival [40].

Interestingly, variation in tumour-accumulated radioactivity in vivo was not as pronounced as would have been expected on the basis of the in vitro data. All of the FR-positive cancer (xeno)grafts were specifically targeted by the folate radiotracer **2**. Most importantly, the in vivo data

obtained with ovarian (IGROV-1, SKOV-3) and cervical (HeLa) cancer cell lines revealed that all showed accumulation in the same range (approx. 1–2% ID/g, 4 h p.i.), but slightly lower than that in KB tumours. Hence, not only the most common KB tumour model, but also clinically relevant tumour types such as ovarian cancer (the primary targets of folate-based diagnosis or treatment), can be successfully targeted by folate-based radiotracers. Data regarding accumulated radioactivity in KB-V1 tumours were also in the same range in vivo. Thus, one could conclude that the ability of KB cells to express FRs and their potential to bind folate radiotracers were retained even in the multiresistant sub-clone KB-V1. It remains to be investigated whether this result can be generalised to other multiresistant cancer cell lines. Such properties of multidrug resistant cancers, which can escape conventional chemotherapy but are revealed to be sensitive to potential folate-based (radio)therapy, are of the highest interest. Even though in vitro colorectal cancers (HT-29, LoVo) showed significantly lower cell accumulation of radioactivity than KB cells, and are therefore supposed to express lower FR levels, the corresponding tumour xenografts revealed reasonable accumulation of radioactivity in vivo.

It is of particular interest for pre-clinical assessment of folate tracers to recognise that each of the tumour models is useful in order to obtain conclusive, reproducible and comparable data, even if tumour masses varied amongst the individual experiments. Fast tumour growth was observed for KB and KB-V1 cells, medium growth for SKOV-3, HeLa, HT-29 and 24JK-FBP cells and only slow tumour growth for Lovo and IGROV-1 cells. In order to assure reliability of the % ID/g data in tumour tissue, a careful analysis of the dependence of tumour mass and % ID was carried out with three of these cell lines, representative for each group: fast- (KB), medium- (24JK-FBP) and slow (IGROV-1)-growing cell lines. The results of this analysis revealed a linear correlation for all three cell lines. Hence, artefacts in our % ID/g values can be largely excluded. In this context, we were also able to demonstrate that the mouse 24JK-FBP cell line is a valuable alternative to human FR-positive cell lines and/or xenografts for folate-based (radio)tracer development. Distribution of the radiotracer in normal tissues of 24JK-FBP tumour graft-bearing C6 black mice was comparable to biodistribution of the radiotracer in athymic nude mice. Thus, mouse strain-dependent differences could be largely excluded. The mouse sarcoma cell line is of particular interest for fast and unproblematic tumour growth, enabling easy practicability and lower cost of in vivo experiments through the use of normal mice. In addition, it should be mentioned, that regarding the FR expression level, 24JK-FBP cells more closely mimic human ovarian cancer cell lines.

Time-dependent in vivo studies, performed with three selected cell lines (KB, IGROV-1 and 24JK-FBP), revealed a desirable fast elimination of radioactivity from the bloodstream. Four hours post injection, radioactivity in the blood pool was less than 0.15% ID/g. These findings are in agreement with the literature, where biological half-lives of several folate conjugates have been reported to be in the range of only a few minutes as a consequence of their rapid renal clearance [16, 41]. Such pharmacokinetics are highly favourable for achieving sufficient contrast between targeted and non-targeted tissues on images.

Taking into account the relatively small variations in total tumour uptake observed in vivo between “high FR-expressing“ (KB) and “low FR-expressing” (LoVo) cell lines, we conclude that FR expression level is not necessarily predictive of the success of FR-targeted radiodiagnosis. However, the findings of this study have to be considered as pre-clinical results. Whether or not this conclusion can be transferred to humans will need to be carefully evaluated. However, if our hypothesis is correct, radiofolates could be of diagnostic potential for virtually all FR-positive tumours.

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