Specific PET imaging of $x_C^{-}$ transporter activity using a $^{18}$F-labeled glutamate derivative reveals a dominant pathway in tumor metabolism

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PET imaging of system $x_C^{-}$ activity

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ABSTRACT

Purpose:

$^{18}$F-labeled small molecules targeting adaptations of tumor metabolism possess the potential for early tumor detection with high sensitivity and specificity by PET imaging. Compounds tracing deranged pathways other than glycolysis may have advantages in situations where FDG has limitations. Aim of the current study was the generation of a metabolically stable $^{18}$F-labeled glutamate analogue for PET imaging of tumors.

Experimental Design:

Derivatives of L-glutamate were investigated in cell competition assays to characterize the responsible transporter. An automated radiosynthesis was established for the most promising candidate. The resulting $^{18}$F-labeled PET tracer was characterized in a panel of in vitro and in vivo tumor models. Tumor specificity was investigated in the turpentine oil induced inflammation model in rats.

Results:

A fluoropropyl substituted glutamate derivative showed strong inhibition in cell uptake assays. The radiosynthesis was established for (4S)-4-(3-[$^{18}$F]fluoropropyl)-L-glutamate (BAY 94-9392). Tracer uptake studies and analysis of knock-down cells demonstrated specific transport of BAY 94-9392 via the cystine/glutamate exchanger designated as system $x_C^-$. No metabolites were observed in mouse blood and tumor cells. PET imaging with excellent tumor visualization and high tumor to background ratios was achieved in preclinical tumor models. In addition, BAY 94-9392 did not accumulate in inflammatory lesions in contrast to FDG.

Conclusions:

BAY 94-9392 is a new tumor specific PET tracer which could be useful to examine system $x_C^-$ activity in vivo as a possible hallmark of tumors to balance oxidative stress. Both preclinical and clinical studies are in progress for further characterization.
Statement of Translational Relevance:

The specific localization and better characterization of tumors in patients remain an important unmet medical need, which hypothetically can be addressed by assessment of system $x_C^\text{-}$ activity. Here we show that a novel $^{18}$F-radiolabeled L-glutamate analogue, BAY 94-9392, is specifically transported via system $x_C^\text{-}$ and can be used for visualization of tumors by PET imaging. It shows high and specific accumulation in tumors as demonstrated in preclinical models. The prevalence and degree of uptake in many different tumor cell lines and tumor xenografts point to the importance of this transport. BAY 94-9392 has the potential to be used clinically for tumor diagnosis due to the high tumor to background ratio and low uptake in normal tissues. In addition, its uptake provides insight into the metabolic requirements of tumors and may provide opportunities to assess detoxification potential and survival pathways to better guide new and existing therapeutic interventions. PET imaging studies using BAY 94-9392 could contribute to elucidate the role of system $x_C^\text{-}$ in oxidative stress and chemoresistance.
INTRODUCTION

Tumors depend on specific adaptations in their intermediary metabolism and efficient detoxification processes to assure survival and proliferation in a challenging microenvironment and during treatment with toxic chemotherapeutic agents. Otto Warburg recognized enhanced breakdown of glucose in tumors compared to normal tissues almost 100 years ago. The increased glycolytic activity observed in many tumors is only one adaptation mechanism, other ones have been subsequently elucidated. For example, aerobic glycolysis in tumors is associated with the lipogenesis and glutaminolytic pathway to provide key intermediates for anabolic reactions. Multiple mechanisms converge to support rapid energy generation, to increase biosynthesis of macromolecules, maintain appropriate cellular redox status, and detoxification potential. Figure 1 provides a simplified scheme on major metabolic pathways in tumors and their interrelationship. Glucose and glutamine are the major carbon and energy sources to be used for growth and proliferation of tumor cells. The contribution of each pathway and the flux rate of breakdown may differ among tumors. High intracellular levels of L-glutamate result from an inwardly directed concentration gradient of L-glutamine and its subsequent deamidation by glutaminase. L-glutamate is further metabolized via three main pathways: (i) during glutaminolysis, (ii) for glutathione (GSH) biosynthesis and (iii) as exchange substrate for system xC. As part of the cellular detoxification system, system xC and GSH play an important role for protection against reactive oxygen species and tumor survival during therapy.

The increased uptake of glucose and its enhanced metabolism is commonly used for tumor imaging with positron emission tomography (PET) using the radiolabeled analogue 2-[18F]fluoro-2-deoxy-D-glucose (FDG). This compound is avidly taken up and phosphorylated by tumors. The substitution at the C-2 position of FDG results in an intracellular trapping of the tracer, since further metabolism and efflux of phosphorylated FDG is hampered. The fate of FDG can be followed non-invasively in vivo by PET imaging and has been implemented as a valuable tool in the clinical routine. Today FDG is by far the most often applied PET tracer worldwide, although it has several pitfalls and limitations. A major limitation of this tracer is posed by the non-specificity towards inflammation. In addition, enhanced uptake of FDG occurs in inflamed tissues around tumors treated with radiation therapy or in scar tissue following surgery, impeding early response assessment. FDG also accumulates in several tissues possessing an increased glucose metabolism such as brain, heart, and brown adipose tissue. Moreover, FDG does not accumulate in tumors with low glycolytic activities, such as prostate cancer, and can not be used for the specific detection of brain tumors and brain.
metastases due to a high background from healthy brain glucose metabolism. To overcome these limitations of FDG, common adaptations of the tumor metabolism beyond enhanced glycolysis need to be exploited to provide an improved PET imaging agent for tumors. Suitable PET tracers might be very helpful to shed more light on those adaptations as they can be directly translated and further investigated in patients for an earlier diagnosis and a better treatment of oncological diseases.

Tumors in general often have to cope with severe conditions of oxidative stress. Thiol containing molecules like the amino acid L-cysteine and the tripeptide glutathione (GSH) are the major cellular components to overcome these conditions and are consumed for detoxification of reactive oxygen species (ROS) and other electrophiles, such as chemotherapeutics. GSH is one of the most abundant intracellular small molecules showing intracellular concentrations in the millimolar range and is considered as the major natural antioxidant. A continuous supply of GSH and its precursors are critical for cell survival and provide a selective advantage for tumor growth. L-cysteine plays a key role as reactive oxygen species scavenger by itself and is the rate-limiting building block for GSH biosynthesis. Furthermore, it is part of the cystine / cysteine redox cycle for maintaining redox balance (figure 1). In the blood the oxidized dimer L-cystine is the dominant form and the availability of L-cysteine is limited. However, L-cysteine can be efficiently provided to cells via the cystine / glutamate exchanger. This transporter now referred to as system xC<sup>+</sup> was first described by Bannai and Kitamura in 1980 as a sodium-independent, high-affinity transporter for L-cystine and L-glutamate in human fibroblasts. System xC<sup>+</sup> is a heterodimeric transporter consisting of two subunits, the light chain xCT (SLC7A11) conferring substrate specificity and the heavy chain 4F2hc (SLC3A2) which is part of several heterodimeric amino acids transporters. SLC3A2 is responsible for their proper membrane localization and activity.

After uptake of L-cystine into cells it is reduced to two molecules of L-cysteine. It is important to note that system xC<sup>+</sup> is not able to discriminate between its natural substrates L-cystine and L-glutamate for the inward directed transport. An increased expression of system xC<sup>+</sup> is found in many tumors, providing a survival advantage by increasing access to L-cysteine via the extracellularly more abundant L-cystine. The concentration of L-glutamate in tumor cells can be as high as 20 mM. This high glutamate concentration is derived primarily from high uptake of L-glutamine and an enhanced glutaminolytic pathway. L-glutamate serves here as a readily available intracellular exchange substrate for the system xC<sup>+</sup> and supports the efficient provision of the sulfur-containing precursor for GSH biosynthesis. In addition to its role as exchange substrate, L-glutamate is either directly consumed in the
glutathione biosynthesis or serves as an anaplerotic substrate to refill the tricarboxylic acid (TCA) cycle intermediate alpha-ketoglutarate (αKG) after transamination (figure 1).

We hypothesized that the enhanced expression and activity of system xC− in tumors represents an attractive opportunity to be exploited for PET tracer discovery. To this end we assessed suitable compounds targeting system xC− for the incorporation of a radioactive PET isotope. The PET radioisotope of choice for radiolabeling of small molecules is fluorine-18 based on its favorable nuclear characteristics and short half-life (t1/2 = 110 min).21 However, some structural prerequisites need to be considered to assure direct radiolabeling with 18F in a high yield for subsequent clinical application. Several structural analogues of L-cystine and L-glutamate are reported to be either substrates or inhibitors for system xC−.17 However, L-cystine itself with its disulfide bond appears sub-optimal for direct and robust 18F-radiolabeling. The xC− inhibitors p-carboxyphenylglycine and sulfasalazine are disadvantageous due to their low binding affinities.17,22 Therefore, we focused our research on substrate compounds such as fluorinated derivatives of L-glutamate. We recently presented promising preclinical and clinical results from 4-[18F]fluoro L-glutamate (BAY 85-8050) for tumor imaging.23,27 This derivative is transported by both, the system xC− and the sodium dependent glutamate transporters from the EAAT family. Though intriguing tumor uptake was seen, the compound was defluorinated in humans resulting in sub-optimal images. For assessment of system xC− activity a derivative is needed that is solely transported via this system. The aim of the present study is to explore the underlying biology and transport mechanisms in more detail and to provide a metabolically stable PET tracer.
MATERIALS AND METHODS

General

The tumor cell lines were obtained from ATTC (USA) or DSZM (Germany) if not otherwise indicated and maintained according to protocols provided by the supplier. All chemicals were obtained from Sigma Aldrich (Taufkirchen, Germany), Tocris Bioscience (Bristol, UK), Alexis Biochemical (now Enzo Life Sciences, Loerrach, Germany), Bachem AG (Bubendorf Switzerland), Toronto Research Chemicals Inc. (North York, Ontario, Canada), Acros Organics (part of Thermo Fisher-Scientific, Nidderau, Germany). [3,4-^3^H]L-glutamate (1.85 TBq / mmol) was purchased from Perkin Elmer (Rodgau, Germany) and [1-^14^C]L-cystine (3.7 GBq / mmol) was obtained from American Radiolabeled Chemicals via Biotrend (Cologne, Germany).

Chemistry & Radiochemistry

The nosylate precursor di-tert-butyl (4S)-N-(tert-butoxycarbonyl)-4-(3-{{(4-nitrophenyl) sulfonyl}oxy}propyl)-L-glutamate was obtained in three steps starting from di-tert-butyl-N-(tert-butoxycarbonyl)-L-glutamate. Radiolabeling of BAY 94-9392 was accomplished by nucleophilic substitution using K^{18}F kryptofix complex and subsequent acidic deprotection followed by cartridge purification. More detailed chemistry information can be found in the supplementary information file and was published recently.

In vitro tracer uptake and competition studies

For tracer uptake and competition studies the tumor cells were seeded in 48 well plates at appropriate concentrations. The cell number used for seeding was adjusted for every tumor cell line to yield approximately 100,000 cells at the day of uptake study. Cells were usually grown for 2-3 days under standard conditions (37 °C, 5% CO\(_2\)) until sub-confluency. The cell number at the day of the uptake assay was determined by detaching cells in three representative wells and cell counting in a Neubauer cell chamber. Uptake data were normalized to 100,000 cells. Prior to the radioactive uptake assay the cell culture medium was removed and the cells were washed twice with PBS buffer. Radiotracers were added to the assay buffer (PBS/ 0.1 % w/v BSA) using 250 kBq/well for \(^{18}\)F-labeled compounds, 37 kBq/well for \(^3\)H-L-glutamate and 3.7 kBq/well for \(^{14}\)C-L-cystine. For competition experiments the cells were co-incubated with competitors either in excess at 1 mM or in a dose dependent manner (0.2 µM - 1 mM). Tracer uptake was stopped by removal of the assay buffer at the time points indicated. Cells were quickly washed twice with PBS and lysed by
the addition of 1N NaOH. The cell lysate was removed from the plates. Radioactivity of $^{18}$F-samples was determined in a gamma counter (Wizard 3, Perkin Elmer, USA). $^{14}$C and $^3$H containing samples were measured in a beta counter (TriCarb 2900TR, Perkin Elmer, USA) after addition of a liquid scintillation cocktail. For transstimulation assays cells were loaded with BAY 94-9392 for 30 min, washed and incubated with compounds at 1 mM for 30 min in PBS. Cell associated and radioactivity released in the supernatant were measured in a gamma-counter.

Generation of xCT knock-down cells

A549 human lung adenocarcinoma cells for xCT knock-down experiments were obtained from ECACC and maintained in DMEM/Ham’s F12 plus L-glutamine (PAA Laboratories, Austria) supplemented with 10% fetal calf serum (PAA Laboratories, Austria). A549 cells were transduced with lentiviruses encoding shRNAs to human xCT mRNA sequence and to a “scramble” non-targeting sequence as a control. shRNA fragments were cloned in a lentivirus vector under the control of a U6 promoter (ViraPower Gateway system from Invitrogen, USA) and carrying puromycin as antibiotic resistance marker. shRNA sequences were selected from human xCT mRNA NM_014331. The corresponding shRNA numbers refer to the position on NM_014331 sequence:

shRNA-1487: - AGTTGCTGGGCTGATTTAT -
shRNA-7701: - TCATACTCGACTAGAAACG -

As non-silencing control shRNA the scramble sequence from QIAGEN (QIAGEN GmbH, Germany) was cloned in the same lentivirus plasmid backbone. A549 cells were seeded on a 6-well plate (200,000 cells/well) for lentivirus transduction. 24 h later the culture medium was replaced with the lentivirus-containing medium (virus concentration 1 µg/ml) and incubated for 6 h. Upon virus transduction the incubation medium was removed and fresh culture medium was added to the cells. 72 h after transduction puromycin-containing medium (1 µg/ml) was added to the cells. After completion of selection the cell samples were collected and xCT silencing was monitored by qRT-PCR. Total RNA was purified from cell lysates with RNeasy mini kit (QIAGEN GmbH, Germany) according to manufacturer’s instructions. cDNA synthesis was performed with reagents and devices from Applied Biosystems. Quantitative RT-PCR based on TaqMan method was applied to determine xCT mRNA level in the cell samples. xCT mRNA level was normalized to HMBS mRNA (hydroxymethylbilane synthase) as internal control.
Gene expression assay for human xCT: Hs00204928_m1 (Applied Biosystems, USA)
Gene expression assay for human HMBS: Hs00609297_m1 (Applied Biosystems, USA)

**In vivo animal studies**

All animal experiments were performed in compliance with the current version of the German law concerning animal protection and welfare. PET imaging and biodistribution experiments were performed with tumor bearing mice or rats. NMRI nude mice (Taconic, Ry, Denmark) or nude rats (RH-Foxn1 nu/nu; Harlan-Winkelmann, Borchen, Germany) were used for subcutaneous inoculation of human xenografts. Female Fischer rats were obtained from Charles River (Sulzfeld, Germany) and used for the combined tumor and turpentine oil inflammation model. For this model the syngenic rat GS9L glioblastoma cell line was used.

Tumor cells (2-5 x 10^6 cells per mouse) were injected subcutaneously and allowed to grow for 1 - 4 weeks. The tumor size at time of the animal study was in the range of 60 to 200 mg. For biodistribution studies 185 kBq of BAY 94-9392 in 100 µL PBS buffer were injected intravenously in conscious animals via the tail vein. Food and water was available ad libitum. Animals were sacrificed at various times (0.25 – 2 h; n = 3 for each time point). Organs and tissues of interest were collected and weighed. The amount of radioactivity was determined with the gamma-counter to calculate uptake as the percentage of injected dose per gram of tissue (%ID/g). The mean %ID/g value and the standard deviation were calculated from 3 animals for each time point.

PET imaging studies with tumor bearing rats were performed using the Inveon small animal PET/CT scanner (Siemens, Knoxville, TN). Nude rats (RH-Foxn1 nu/nu; Harlan-Winkelmann, Borchen, Germany) were inoculated with 5 x 10^6 NCI-H460 tumor cells in 100 µL matrigel in the right flank. 10 – 16 MBq of BAY 94-9392 were injected intravenously in conscious animals via the tail vein. After 85 min post injection the animals were anesthetized with isoflurane (Abbott) and the PET data were acquired from 90 to 110 min p.i.. Rat GS9L glioblastoma tumor cells (2 x 10^6 cells in a volume of 100 µL of medium + matrigel) were inoculated subcutaneously into the right hind leg. 5 days post GS9L tumor cell inoculation all animals received an injection of 100 µL turpentine oil in the left calf muscle. PET imaging using either FDG or BAY 94-9392 was performed 72 h post induction of the inflammation. No fasting was applied for both groups prior to tracer injection. Animals receiving FDG were kept anaesthetized with isoflurane and warmed for the whole distribution period to avoid confounding tracer uptake in muscle tissue due to locomotor activity. PET data from both groups were acquired from 60 - 70 min post injection. After finishing PET
data acquisition the animals were sacrificed, the inflamed muscle and contralateral muscle tissue were removed, weighed and radioactivity was determined with a gamma-counter to calculate the uptake as the percentage of injected dose per gram of tissue (%ID/g). The mean %ID/g value and the standard deviation were calculated from 3 animals, each. For subsequent histopathological analysis muscle samples were formalin fixed and paraffin embedded. H&E staining and immunohistochemistry using the CD68 antibody (Serotec MCA341R) for macrophage staining were performed using established standard protocols.
RESULTS

In vitro studies: Identification of BAY 94-9392

Substituted L-glutamate derivatives were investigated in cell competition assays to gain insights in structure-activity relationships of the involved transporter(s). These non-radioactive compounds were tested in large molar excess for their ability to compete with the uptake of a previously described radioabeled L-glutamate derivative.\(^{27}\) Competition data are summarized in Table 1. Co-incubation of the radiotracer with L-glutamate and L-cystine strongly reduced the tracer uptake to 9.9 % and 9.7 %, respectively, pointing to the involvement of system xC\(^{-}\). No competition (95.6 % uptake of control) was determined by co-incubation with 2-ethyl-glutamate. Substitution in 3-position reduced radiotracer uptake to 51.8 % or 33.7 % when incubated with 3-hydroxyl-glutamate or (+/-)-threo-3-methyl glutamate, respectively. 4-substituted glutamate derivatives showed high competition (5.4 - 33.5 % uptake). 4S-configurated methyl and hydroxyl derivatives showed higher competition (7.7 % and 13.2 %) compared to the 4R-configurated derivatives (29.5 % and 33.5 %). Strong competition was observed with the 4S-fluoropropyl derivative \(^{[19F]}\)-BAY 94-9392 (5.4 %).

\(^{[19F]}\)-BAY 94-9392 was further studied in a dose dependent manner for competition against the natural radiolabeled substrates of system xC\(^{-}\), \(^{[3H]}\)L-glutamate and \(^{[14C]}\)L-cystine. IC\(_{50}\) values of 29.1 and 33.6 \(\mu\)M were determined for \(^{[19F]}\)-BAY 94-9392 (figure 2a,b). These values are lower than the values determined for L-glutamate and L-cystine (111.7 \(\mu\)M and 116.4 \(\mu\)M, respectively).

To get access to the \(^{18F}\)-labeled fluoropropyl derivative, an automated radiosynthesis was established using a nosylate precursor. (4S)-(3-[\(^{18F}\)]fluoropropyl)-L-glutamate (BAY 94-9392) was obtained in a radiochemical yield of > 40 % decay corrected (d.c.) and purity of > 92 % (n = 40). For more details see supplementary data.

In vitro studies: Characterization of BAY 94-9392 uptake and retention

The uptake of BAY 94-9392 was investigated in different tumor cell lines. Time dependency of uptake is shown in figure 2c for the human lung tumor cell line NCI-H460. An equilibrium was reached after 60 min of incubation at which time approx. 16 % of the applied dose was internalized. Representative tumor cell lines were investigated and showed an uptake with values ranging from 1 - 18 % uptake per 100,000 cells in 30 min (figure 2d). Highest uptake was observed in the human NSCLC cell line NCI-H322 with 18.3 % per
100,000 cells. Prostate cancer 22RV1 cells and liver cancer HepG2 showed lower uptake (1.7 % and 1.4 % uptake per 100,000 cells, respectively).

To further characterize the responsible transporter for cellular uptake of BAY 94-9392, tracer uptake was investigated in the presence of structurally similar compounds. Strong inhibition of BAY 94-9392 uptake (~80 %) was observed by co-incubation with an excess of either L-glutamate, L-cystine or the system xC⁻-specific inhibitor p-carboxy-phenylglycine (CPG), but not by L- or D-aspartate. The competition profile for NCI-H460 cells is shown as an example in figure 3a and indicates specific uptake through system xC⁻. To further demonstrate that uptake of BAY 94-9392 proceeds via this transport system knock-down cells were generated using the human A549 lung tumor cell line. mRNA levels of the xCT subunit of system xC⁻ were reduced to 14 - 18 % in the A549 cell sub clones carrying two independent shRNA sequences, sh1487 and sh7701, respectively. Knock-down cells were viable and showed slightly reduced proliferation (44 - 56 %) compared to the parental cell line (see supplementary information). BAY 94-9392 cell uptake was reduced to 29.7 and 38.0 % compared to mock treated cells. Thus, xCT knock down reduces the capacity for initial uptake of BAY 94-9392 via system xC⁻ (figure 3b).

The intensity of a tumor PET signal in vivo depends on rapid and high uptake as well as strong retention. In order to study intracellular retention and metabolism, BAY 94-9392 loaded cells were investigated under efflux conditions. In PBS buffer containing no transporter substrates ~80 % of the initial tracer uptake is retained in tumor cells. Incubation with PBS buffer containing excess of L-glutamate, ¹⁹F-BAY 94-9392 or complete MEM medium with L-cystine caused release of intracellular radioactivity within 30 min. High retention was observed after incubation of the cells with MEM plus mercaptoethanol (ME) or with PBS containing the inhibitors of system xC⁻, CPG or sulfasalazine. (figure 3c). Thin-layer chromatography (TLC) of the cell lysates and released radioactivity in the supernatant revealed exclusive presence of the parent compound. (data not shown)

**In vivo studies – Pharmacokinetic and tumor targeting**

Tumor uptake and biodistribution of BAY 94-9392 were assessed in tumor-bearing animals. After tracer injection in conscious animals, the animals had access to water and food ad libitum. During the distribution period anesthesia was not necessary for BAY 94-9392 whereas for FDG anesthesia is recommended to avoid its high uptake in muscle and brown fat.²⁸ The results are expressed as percentage of the injected dose (% ID) and are normalized for tissue weight in gram. Quantitative and kinetic analysis of the BAY 94-9392 uptake in
NCI-H460 tumors in mice reveal a rapid and high initial tumor uptake which remains constant at a high level (4.1 ± 1.4 % ID/g at 0.25 hr p.i. and 3.2 ± 0.4 % ID/g at 1 hr p.i.). Rapid blood clearance via the kidneys was observed resulting in a high tumor-to-blood ratio of 14 and a tumor-to-muscle ratio of 27 at 1 hr p.i.. The pancreas showed an initial uptake of 19.4 ± 4.2 % ID/g at 0.25 hr p.i. decreasing to 5.8 ± 0.9 % ID/g at 1 hr p.i.. Other organs of interest such as the brain, liver and muscle showed negligible or low uptake (<1 % ID/g) (figure 4a). The bone signal remains <1 % ID/g over time indicating no defluorination of BAY 94-9392. The analysis of potential metabolites of BAY 94-9392 was performed in blood samples at different time points. Only the parent compound was detectable by TLC analysis (see also supplementary information). The tumor uptake of BAY 94-9392 was investigated in a panel of other subcutaneous human tumor models in mice. Most of the tumors showed an uptake in the range of 2 - 4 % ID/g at 60 min p.i. (figure 4b). These tumor uptake values together with low background signals and rapid clearance provide high contrast for tumor imaging. Comparable or slightly higher tumor uptake values were observed for FDG in animal models. However, FDG is very sensitive for study conditions and some prerequisites need to be considered. For example, if mice were kept under anesthesia and warmed after tracer injection, NCI-H460 tumors in mice showed a FDG uptake of 4.0 ± 0.4 % ID/g yielding a tumor to blood ratio of 8. If no anesthesia was applied, the FDG uptake in NCI-H460 tumors dropped to 1.8 ± 0.2 % ID/g whereas other organs and tissues like muscle and brown fat showed increased uptake. (unpublished own data). In contrast, for BAY 94-9392 no influence of animal handling conditions such as anesthesia and locomotor activity was observed on tumor uptake.

**In vivo studies: PET imaging**

PET/CT imaging was performed with BAY 94-9392 in human NCI-H460 tumor bearing rats. Almost exclusive tracer accumulation was observed in the tumor, kidney and the pancreas relative to all other normal tissues (figure 4c). FDG was investigated under analogous conditions and showed higher background signals arising from its uptake in muscle, brain, intestine, brown fat and other tissues (see also supplementary data).

To test whether inflammatory processes would also accumulate BAY 94-9392 the tracer uptake was investigated in a combined tumor and inflammation model. Immuno-competent rats were used bearing a rat GS9L glioblastoma subcutaneously in one leg. An inflammatory process was initiated in the contralateral calf muscle using turpentine oil. Both, BAY 94-9392 and FDG showed uptake in the GS9L tumors. However, BAY 94-9392 showed
negligible uptake in inflammatory lesions (0.18 ± 0.02 % ID/g, n=3, figure 5a) compared to the uptake of FDG (1.95 ± 0.18 % ID/g, n=3, figure 5b). Histopathological analysis confirmed similar extent of inflammation with massive invasion of neutrophils and macrophages in all animals (figure 5c).
DISCUSSION

Adaptations of tumor metabolism offer opportunities to provide more specific PET tracers for tumor imaging and to overcome limitations of FDG. In this context, the system \( x_C^- \) plays an important role in tumors by providing an efficient access to L-cystine conferring a selective advantage for growth and survival. Substituted glutamate derivatives were investigated in cell competition assays for their interaction with system \( x_C^- \). It was observed that 4-substituted glutamate derivatives showed high competition with radiolabeled glutamate compared substituted compounds in 2- and 3-position. A 4-fluoropropyl substituted derivative (\(^{19}\text{F}-\text{BAY 94-9392}\)) showed strong competition with low IC\(_{50}\) values. This derivative was chosen based on its high affinity to system \( x_C^- \) and its accessibility for incorporation of a radioactive fluorine-18 atom. An efficient radiosynthesis was established to produce the \(^{18}\text{F}-\text{radiolabeled derivative BAY 94-9392}\) which can be used to trace its transport and retention in tumor cells as well as for tumor PET imaging. Time dependent uptake was observed that reached a plateau after 60 min, indicating a transporter mediated uptake. In general, a high uptake of BAY 94-9392 was observed in several tumor cell lines from different entities with variations in the absolute amounts. Specific transport of BAY 94-9392 via system \( x_C^- \) was revealed by strong competition of tracer uptake with L-glutamate and L-cystine as well as with the xCT-subunit specific inhibitor CPG. Only minor competition was observed with either D- and L-aspartate which are both substrates along with L-glutamate for the sodium-dependent excitatory amino acid transporter family, i.e., EAAT 1-5. In addition, the specificity of BAY 94-9392 transport and dependency of system \( x_C^- \) expression level was investigated using two different stable knock down cell clones of the xCT-subunit from the A549 lung cancer cell line. Reduced mRNA levels of xCT led to a reduced capacity for uptake of BAY 94-9392.

A strong intracellular retention of BAY 94-9392 was observed in PBS buffer in the absence of putative exchange substrates for system \( x_C^- \) indicating no further involvement of other efflux mechanisms. However, in the presence of excess transporter substrates such as L-glutamate, L-cystine or \(^{19}\text{F}-\text{BAY 94-9392}\) in the efflux buffer a complete exchange of the intracellular glutamate can be observed as traced by an almost complete release of intracellular radioactivity. Obviously, no intracellular metabolism of BAY 94-9392 occurs and therefore it can exit the cells only via system \( x_C^- \). TLC analyses confirmed the presence of the parent compound in the cell lysates and supernatants as the sole component. Taken together, these \textit{in vitro} experiments clearly indicate that even without an intracellular enzymatic conversion a high retention in tumor cells can be achieved. This is markedly
different from FDG’s cellular retention in which its phosphorylation is required to prevent subsequent efflux.²⁹

To test the hypothesis that system $x_C^-$ activity is an important pathway in tumors compared to other tissues, biodistribution as well as PET imaging studies of tumor bearing animals were performed. The rapid and high tumor uptake of BAY 94-9392 together with a rapid clearance from blood and other healthy tissues led to excellent tumor visualization for up to 2 h p.i.. Broad applicability of this PET tracer for tumor imaging was demonstrated by analysis of other human tumor models in mice. Even tumors with rather low total uptake values, such as A549 (1.6 % ID/g at 60 min p.i.) can be well visualized by PET in mice (data not shown) due to the favorable low background.

For a better understanding of the sustained BAY 94-9392 tumor signal the source and metabolic fate of intracellular L-glutamate needs further consideration. The blood concentration of L-glutamate is approximately 50 µM and similar to L-cystine concentration.³⁰,³¹ However, high intracellular concentrations of L-glutamate up to 20 mM have been reported in tumors.¹⁹ The majority of intracellular L-glutamate is derived from L-glutamine which is the most abundant amino acid in the blood. It is well known that proliferating cells have a high demand for this amino acid. Efficient supply of L-glutamine in tumors is mediated by a set of different transporters, in particular ASCT2 (SLC1A5).³² The high uptake of L-glutamine and its subsequent deamination by glutaminase form the basis for a high intracellular concentration of glutamate. Recently, data were published about the radiosynthesis of $^{18}$F-glutamine derivatives to study this process.³³ The carbon backbone of glutamine is similar to glutamate, but different transporters and a different metabolic pathway are being addressed. As several anabolic pathways, in particular the lipogenesis pathway, are responsible for depletion of TCA cycle intermediates there is a high demand for anaplerosis which can be met in part by L-glutamate. The high intracellular concentration of L-glutamate in tumors assures constant availability of an exchange substrate and promotes system $x_C^-$ operation at a high activity. The dependency of $x_C^-$ activity on the intracellular glutamate concentration was studied with human fibroblasts using $[^3]$H-L-cystine as radiotracer. Fibroblasts were incubated in glutamine / glutamate free medium for up to 24 h. Glutamate levels dropped within 3 h which was paralleled by a reduced rate of $[^3]$H-L-cystine uptake.¹⁶ However, our in vivo experiments showed high uptake of BAY 94-9392 in multiple tumor models and a rather constant tumor signal over time. This indicates the presence of a high $x_C^-$ activity along with a robust and high intracellular concentration of glutamate as a common feature of tumors. Thus, BAY 94-9392 gets diluted in the large intracellular glutamate pool.
conferring tracer retention in the cells without need for enzymatic processing. Only if an excess of extracellular exchange substrates is provided to the tracer loaded cells, a complete exchange of intracellular glutamate can be achieved concomitant with a release of BAY 94-9392.

A critical issue is the understanding of the relative role of transporter expression and changes of flux in tumor cells. Comprehensive studies to correlate the uptake and retention of BAY 94-9392 with mRNA levels of system xC−, the transporter (subunit) density and activity at protein level as well as glutamate concentrations are currently ongoing. Previously published data indicate the importance of system xC− for mediating chemoresistance of melphalan, cisplatin, irinotecan and celastrol.34,35,36,37,38 In addition, an interaction of the xCT subunit of system xC− with a variant of the cancer stem cell marker CD44 was published recently. CD44v contributes to the regulation of the redox status in tumors by stabilizing the xCT subunit and promotes tumor growth.39 Ablation of CD44v induced loss of xCT from the cell surface and suppressed tumor growth in a transgenic mouse model of gastric cancer. In future studies BAY 94-9392 might not only be used for more specific tumor detection, but can be employed as novel tool for the non-invasive assessment of tumors redox balance and thiol-mediated detoxification potential.

In addition to its role in the tumor metabolism, elevated expression of system xC− has been reported in activated macrophages in vitro but not in tumor-associated macrophages.40, 41 The turpentine oil inflammation model has been used extensively to model sterile inflammation and accumulation of macrophages is reported.42 However, no accumulation of BAY 94-9392 was observed in histopathologically proven inflammatory lesions containing massive invasion of neutrophiles and macrophages. Thus, the tracer uptake in neutrophils and macrophages via system xC− does not appear to be a dominant mechanism in this particular in vivo inflammation model.

In conclusion, system xC− is an important transporter in tumor cells. Its critical role in malignant transformation can be exploited to develop imaging and therapeutic agents. The 18F-radiolabeled glutamate derivative BAY 94-9392 is a metabolically stable PET tracer in rodents. It is specifically taken up via system xC− and strongly retained in tumors. PET imaging of system xC− activity using BAY 94-9392 has a potential for not only improving early detection and staging of several tumors with higher sensitivity and specificity than FDG, but also guiding therapeutic interventions by imaging metabolic adaptations to oxidative stress in vivo. More studies are needed to further elucidate and correlate the uptake pattern of BAY 94-9392 with underlying chemoresistance mechanisms. Clinical studies using BAY 94-
are in progress to characterize this compound in cancer patients. Ongoing implementation of such new PET tracers raises hopes of being able to better detect and characterize unique adaptive and metabolic pathways within malignant tissues.
ACKNOWLEDGMENTS

The skillful technical assistance of research staff at Bayer HealthCare Pharmaceuticals, Berlin is gratefully acknowledged. We thank Manfred K. Grieshaber, Institute of Biochemistry, University of Düsseldorf, for stimulating scientific discussions about glutamate metabolism and adaptive biochemical mechanisms. We are grateful to Anna-Lena Frisk for performing immunohistochemistry analyses.
TABLES

Table 1: Structure activity relationship of glutamate derivatives

Uptake of BAY 85-8050 (4-[18F]fluoroglutamate)\textsuperscript{27} in A549 cells in the presence and absence of glutamate derivatives (1 mM) or L-cystine. *L-cystine was studied at 0.8 mM due to its limited solubility. Values are reported as % of control (mean, n=3, +/- SD).
FIGURE LEGENDS

Figure 1. Tumor specific adaptations of intermediary metabolism for assuring growth, proliferation and survival. Tumors are characterized by adaptation of several catabolic and anabolic pathways to meet their demands for energy, growth, proliferation and detoxification. In particular, pathways comprising glycolysis, lipogenesis, glutaminolysis and glutathione biosynthesis are pronounced. The TCA cycle in tumors is mostly truncated leading to the efflux of mitochondrial citrate into the cytosol providing carbons for lipogenesis. Here, L-glutamate serves as the major anaplerotic substrate to refuel the TCA cycle. L-glutamate is obtained from glutamine degradation via glutaminase and accumulates intracellularly at high concentrations. In addition, L-glutamate is one component of the tripeptide glutathione (GSH) and is consumed for its biosynthesis or is used as an exchange substrate for the uptake of L-cystine via the system xC− transporter.

Abbreviations: 1 – hexokinase; 2 – PK-M2: pyruvate kinase – M2 isoform; 3 – enzyme described, but not identified, yet; 4 – malic enzyme; 5 – glutaminase; 6 – glutamate transaminases (GOT/GPT) and/or glutamate dehydrogenase; 7 – aconitase; 8 – lipogenesis pathway: ATP-citrate lyase, acetyl CoA carboxylase, fatty acid synthase; 9 – thioredoxin reductases; 10 – gamma-glutamyl cysteine synthetase; 11 – pyruvate carboxylase; amino acid transport systems: system ASC – comprising for example ASCT2 (SLC1A5); system L – heterodimer consisting of LAT1 (SLC7A5) and 4F2hc (SLC3A2) subunits; system xc− – heterodimer consisting of xCT (SLC7A11) and 4F2hc (SLC3A2) subunits; Cit – citrate; EAA – essential amino acids; Glc – glucose; Glu – glutamate; Gln – glutamine; αKG – alpha-ketoglutarate; Lact – lactate; Mal – malate; MRP – multi-drug resistance proteine; OAA – oxaloacetic acid; PEP – phosphoenol pyruvate; Pyr – pyruvate.

Figure 2. In vitro characterization of (4S)-(3-fluoropropyl)-L-glutamate and its 18F-radiolabeled derivative BAY 94-9392.

(a, b) Dose dependent competition cell uptake assays were performed in human NCI-H460 lung cancer cells using the 19F-derivative of BAY 94-9392 and the radiolabeled natural xCT substrates [3H]L-glutamate and [14C]L-cystine. (c) Time dependent uptake of BAY 94-9392 in NCI-H460 lung tumor cells was monitored for 120 min. (d) Overview of representative tumor cell lines used for BAY 94-9392 cell uptake studies. Uptake of BAY 94-9392 was determined after 30 min incubation.
Figure 3. Identification and characterization of the transporter responsible for cellular uptake of BAY 94-9392.

(a) Co-incubation of NCI-H460 cells was performed with BAY 94-9392 as $^{18}$F-radiotracer and several structural similar compounds in excess (1 mM) for 10 min in PBS buffer. Strong inhibition of tracer uptake was observed with L-glutamate, L-cystine and the xCT-subunit inhibitor p-carboxyphenylglycine (CPG) and the non-radioactive derivative $^{19}$F-BAY 94-9392 indicating uptake through system xC$. Negligible competition was observed with L- and D-aspartate confirming no or minor involvement of glutamate transporters from the excitatory amino acid transporter family. (b) xCT shRNA knock-down cells were generated using the human A549 lung cancer cell line. Mock treated A549 cells and the xCT knock-down clones sh1487 and sh7701 were investigated for uptake of BAY 94-9392 and a reduced uptake was determined. (c) Intracellular retention of BAY 94-9392 was investigated in a trans-stimulation assay. NCI-H460 cells were loaded with BAY 94-9392 for 30 min. Cells were washed and re-incubated with different compounds at 1 mM concentration. Intracellular and the released radioactivity into the supernatant were measured. Strong release of radioactivity was observed only in the presence of excess amounts of putative substrates for system xC$. Inhibitors of system xC$ such as CPG (p-carboxyphenylglycine) or sulfasalazine are not able to release activity from BAY 94-9392-loaded cells.

Figure 4. Analysis of pharmacokinetic, biodistribution and tumor targeting of BAY 94-9392.

(a) Biodistribution and pharmacokinetic of BAY 94-9392 were analyzed in NCI-H460 tumor bearing mice. The time course of BAY 94-9392 signal is shown for tumor as well as for selected organs and tissues. A sustained tumor signal at a high level and rapid clearance from most healthy organs and tissues was determined. Only the kidneys and the pancreas showed still increased levels of BAY 94-9392 at later time points. (b) Tumor uptake of BAY 94-9392 was investigated in a panel of other subcutaneous human tumor models in mice. Tracer uptake at 60 min p.i. is displayed. Most tumors showed an uptake level between 2 – 4 % ID/g. (c) Human NCI-H460 lung tumor bearing rats were used for tumor visualization by PET. Surface rendered whole body PET data (in yellow-red) were acquired from 90 - 110 min p.i. and fused with the CT image (in grey). A strong tumor signal and low background from healthy tissues were confirmed by PET. In addition to the tumor signal, only the kidneys and the bladder as
excretion organs and the pancreas are visible. A rotating image is available in the supplemental information.

**Figure 5. Investigation of BAY 94-9392 and FDG in a combined rat GS9L glioblastoma and inflammation model by PET/CT imaging in rats.** PET imaging was performed 72 hr after turpentine oil injection with either BAY 94-9392 or with FDG. Whole body PET data (*colored*) were acquired from 60 – 70 min p.i. and fused with the CT image (*in grey*) *(a)* BAY 94-9392 showed strong accumulation in GS9L tumors but no detectable uptake in the inflammatory lesions. *(b)* FDG accumulates in both, the tumor and inflammation. In addition, a higher uptake was observed for FDG in the gastrointestinal (GI) tract compared to BAY 94-9392. *(c)* Histopathological analysis of the inflamed muscle samples showed presence of central necrosis with destructed muscle tissue and massive invasion of neutrophils (*top*). CD68 immunohistochemistry was performed to stain macrophages (*bottom*).
REFERENCES


38 Pham AN, Blower PE, Alvarado O, Ravula R, Gout PW, Huang Y. Pharmacogenomic approach reveals a role for the x(c)- cystine/glutamate antiporter in growth and celastrol resistance of glioma cell lines. J Pharmacol Exp Ther 2010;332:949-58.


<table>
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<th>Compound (1 mM)</th>
<th>Structure</th>
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Figure 1

Glu / Cystine

Cysteine / cystine redox cycle

Building Blocks

Tumor Cell

Growth & Proliferation

Lipogenesis

Glycolysis

Glutamine

Glutaminolysis

Glutathione Biosynthesis

ROS Protection & Survival

Nucleotides & Building Blocks

Growth & Proliferation
Figure 2

(a) 

[Graph showing uptake of \(^{18}F\)-BAY 94-9392 and L-Glutamate in cells against competitor [mM] from 0.0001 to 10.

(b) 

[Graph showing uptake of \(^{14}C\)-cystine in cells against competitor [mM] from 0.0001 to 10.

(c) 

[Graph showing uptake of \(^{4}H\)-BAY 94-9392 in cells over time from 0 to 120 minutes.

(d) 

[Bar graph comparing uptake of \(^{18}F\)-BAY 94-9392 in various cell lines such as H460, A549, NCI-H292, NCI-H322, PC3, 22RV1, SK-OV3, HepG2, GSGL, and Capan, with lung ca, prostate ca, ovary ca, liver ca, glioma, and pancreatic ca indicated by different shading.

Research on October 15, 2017. © 2011 American Association for Cancer Research. Manuscripts have been peer-reviewed and accepted for publication but have not yet been edited.
Figure 3

a

![Graph showing uptake of various compounds compared to control.]

b

![Graphs showing xCT mRNA expression and BAY 94-9392 uptake.]

c

![Graph showing influx and efflux of compounds.]

---

**Legend:**
- Influx: 10 min
- Block 1 mM
- 19F-BAY 94-9392 uptake
- Control
- PBS
- L-Glutamate
- L-Cystine
- CPG
- D-Glutamate
- L-Aspartate
- D-Aspartate
- 19F-BAY 94-9392
- xCT mRNA (normalized to HMBS)
- A549 (mock)
- A549 sh1487
- A549 sh77701
- Influx: cells after efflux
- Supernatant

---

% activity

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Figure 4

(a) Tumor uptake [% ID/g] for different organs over time [min].

(b) Tumor uptake [% ID/g] at 60 min p.i. for various cell lines.

(c) PET/CT imaging showing uptake in different organs.
Figure 5

(a) Inflammation (not detectable)

(b) GI tract

(c) Tumor
Clinical Cancer Research

Specific PET imaging of $x_C^-$ transporter activity using a $^{18}$F-labeled glutamate derivative reveals a dominant pathway in tumor metabolism


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