SUPPLEMENTAL INFORMATION

PET IMAGING OF GLUTAMINOLYSIS IN TUMORS BY [^{18}F](*2S,4R*)4-FLUOROGLUTAMINE

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Radiolabeling of [^{18}F](2S,4R)4-fluoroglutamine

The radiosynthesis was performed by a method as described previously (*1*). Briefly, an activated SepPak® Light QMA Carb was loaded with ^{18}F fluoride ion (740 to 1480 MBq (20 to 40 mCi)) and eluted with 1 mL 18-crown-6/$KHCO_3$ (160 mg 18-Crown-6 in 18.6 mL ACN/29 mg $KHCO_3$ in 3.4 mL water). The solution was blown to dryness with argon and dried twice azeotropically with 1 mL acetonitrile at 80 ºC under a flow of argon. The dried ^{18}F was cooled in an ice bath and 5 mg of tosylate precursor ((2S,4R)-tert-butyl-2-(tert-butoxycarbonylamino)-5-oxo-4-(tosyloxy)-5-(2,4,6-trimethoxybenzylamino)-pentanoate) was dissolved in 0.5 mL acetonitrile and added to the dried ^{18}F fluoride ion. The mixture was heated for 15 min at 70 ºC oil bath. The mixture was cooled in ice bath, 0.5 mL acetonitrile and 8 mL water were added. The mixture was loaded onto an activated Oasis® HLB 3cc cartridge, pushed through and washed twice with 3 mL water. The desired radiolabeled compound was eluted with 0.5 mL ethanol (~ 10% of the total radioactivity). The ethanolic solution was blown to dryness, cooled in an ice bath and a mixture of 595 mL TFA/5 mL anisole was added. The solution was heated for 5 min at 60 ºC. TFA and anisole were removed while still warm under a stream of argon; the residue was taken into 1 mL DPBS buffer; the solution was filtered through a 0.45 micrometer filter to yield the desired radioactive [^{18}F](2S,4R)4-fluoroglutamine. The radiochemical and stereochemical purity was determined by chiral HPLC (Chirex 3126 (d)-penicillamine, 1 mM $CuSO_4$ solution, 1 mL/min, retention time for the (2S,4R) isomer ~11 min, the (2R,4R) isomer ~18 min) (RCY (non-decay corrected) 8.4 ± 3.4%; RCP 98 ± 1%; optical purity > 91 ± 8%; n = 10).

Cell culture

9L cells (ATCC, Manassas, VA), SF188 cells (Neurosurgery Tissue Core, University of California, San Francisco, CA) and Bcl-xL transfected $SF188_{bcl-xl}$ were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hycolne, Logan, UT) and 1% penicillin/ streptomycin. Cells were maintained in T-75 culture flask under humidified incubator conditions (37 ^{o}C, 5% CO_2) and were routinely passaged at confluence.

In vitro cell uptake studies

The cellular uptake of [^{18}F](2S, 4R)4-fluoroglutamine was studied in 9L, SF188 and $SF188_{bcl-xl}$ glioma cell lines. [^{18}F]FDG and L-[3,4-^{3}H(N)]-glutamine (specific acitivity 1.11-2.22 TBq/mmol and >97% purity) purchased from IBA molecular (Dulles, VA) and Perkin Elmer (Waltham, MA) respectively, were used as reference ligands.

Tumor cells were plated (2.0×10^5 cells/well) 24 hours in the media prior to ligand incubation. On the day of experiment, the media was aspirated and the cells were washed 3 × with 1 mL with warm phosphate buffered saline (PBS, containing 0.90 mM of Ca^{2+} and 1.05 mM of Mg^{2+}). [^{18}F](2S,4R)4-fluoroglutamine, [^{18}F]FDG and the L-[3,4-^{3}H(N)]-glutamine ([^{3}H]GLN) were dissolved in PBS solution and were added to each well (37 KBq/mL/well) and incubated at 37 °C for 5, 30, 60, 120 minutes. At the end of the incubation period, the wells were aspirated and then the residual cells were washed 3 times with 1 mL ice cold PBS without Ca^{2+} and Mg^{2+}. After washing with cold PBS, 350 mL 1M NaOH was used to lyse the cells. The lysed cells were collected onto filter paper and counted together with samples of initial dose using a gamma counter (Packard Cobra). The cell lysate (100 μL) was used for determination of protein concentration by modified Lowry protein assay. The data was normalized as percentage uptake of initial

dose (ID) relative to 100 μg protein content (% ID/100 μg protein). For dual-isotope studies using [^{18}F](2S, 4R)4-fluoroglutamine or [^{18}F]FDG together with L-[3,4-^{3}H(N)]-glutamine, the vials were counted first using a gamma counter to obtain ^{18}F counts. The filter papers were then placed in scintillation vials and counting fluid was added (7 mL Ecolite+) and the vials were then counted with a beta counter (Beckman, LS 6500) 24 h later.

In vitro transport characterization studies

To characterize the transport of [^{18}F](2S,4R)4-fluoroglutamine, competitive inhibition studies, sodium and pH dependence studies were conducted using 9L cell line. The tracer was incubated at 37 °C for 30 minutes. The cells were processed as described above. In inhibition studies, various inhibitors were added to the cells in concentrations ranging from 0.1 mM to 5 mM in PBS solution. Selected inhibitors included synthetic amino acid transport inhibitors such as N-methyl-α-aminoisobutyric acid (MeAIB) for system A, 2-amino-bicylo[2.2.1] heptane-2-carboxylic acid (BCH) for system L and L-γ-Glutamyl-p-nitroanilide (GPNA) for system ASCT2 (*2-5*). Natural amino acid such as L-Serine and L-Glutamine were also used as inhibitors, although they are not specific for a particular amino acid transport system. They exhibit preference towards system ASC. In sodium dependency studies, PBS buffer was replaced with Na^{+} free solution (143 mM choline chloride, 2.68 mM KCl and 1.47 mM KH_2PO_4). In pH dependence studies, PBS solution was adjusted to desirable pH with NaOH and HCl solution. The data was normalized in reference to uptake of [^{18}F](2S,4R)4-fluoroglutamine without any inhibitor in PBS solution at pH 7.4.

Protein Incorporation

To measure the extent of protein incorporation of [^{18}F](2S,4R)4-fluoroglutamine, protein-bound activity in SF188 and 9L cells was determined at 30 and 120 min after incubation. The cells were incubated with 222 KBq [^{18}F](2S,4R)4-fluoroglutamine or 37 KBq L-[3,4-^{3}H(N)]-glutamine in 3 mL PBS. At the end of incubation, the radioactive medium was removed, the cells were washed three times with ice cold PBS without Ca^{2+} and Mg^{2+}, treated with 0.25% trypsin and resuspended in PBS. The samples were centrifuged (18,000 g, 5 min), the supernatant removed and the cells were suspended in 200 μL 1% Triton-X 100 (Sigma, St.Louis, MO). After vortex, 800 μL of ice cold 15% trichloroacetic acid (TCA) was added to the solution. After 30 min precipitation, the cells were centrifuged again (18,000 g, 15 min) and washed twice with 15% ice cold TCA. The radioactivity in both supernatant and pellet was determined. Protein incorporation was calculated as percentage of acid perceptible activity. Cells incubated in the medium containing 5 μg/mL cycloheximide were used as negative control and the precipitation of intracellular [^{18}F](2S,4R)4-fluoroglutamine in its free form was monitored by calculating the recovery of activity added just before TCA precipitation.

In vivo biodistribution study in normal ICR male mice

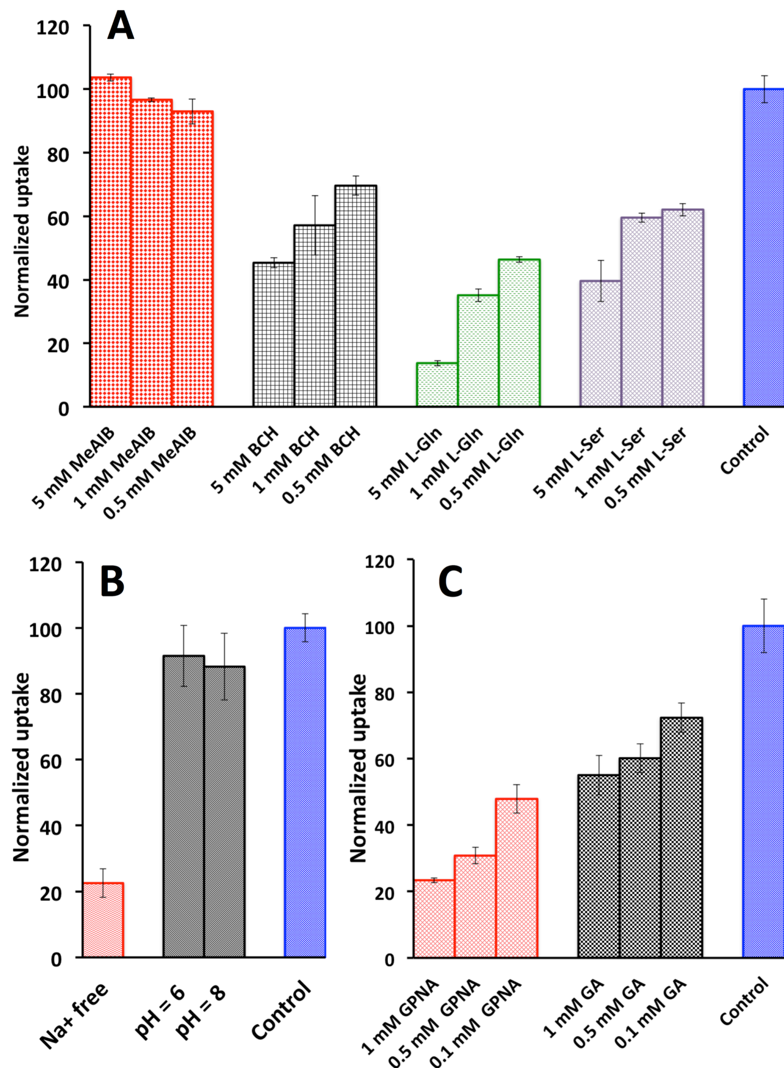
To test the [^{18}F](2S,4R)4-fluoroglutamine as a tumor PET imaging agent, we first tested this tracer in normal ICR mice (20 - 25 grams). 5 mice per group were used for the biodistribution study. The mice were put under anesthesia with the use of isoflurane (2 - 3%) and 0.15 mL saline solution containing 925 KBq of [^{18}F](2S,4R)4-fluoroglutamine was injected via the lateral tail vein. The mice were sacrificed at 2, 30, 60, 120 and 240 minutes post-injection by cardiac excision while under isoflurane anesthesia. The organs of interest were removed, weighed and the radioactivity was counted with a gamma

counter (Packard Cobra). The percent dose per gram was calculated by a comparison of the tissue activity counts to counts of 1.0 % of the initial dose. The initial dose consisted of 100 times diluted aliquots of the injected material measured at the same rate (0.5 min/sample, 80% efficiency).

In vivo biodistribution study in Fischer 344 rats bearing 9L tumors

Fischer 344 rats were purchased from Charles River Laboratories (Malvern, PA). 9L cells (rat brain gliosarcoma) were cultured with the use of DMEM, fetal bovine serum and 100 units/mL Penicillin, 100 µg/mL Streptomycin. Cells were detached and spun down at 200 g for 5 minutes. Cells were resuspended in phosphate buffered saline (PBS, containing 0.90 mM of Ca^{2+} and 1.05 mM of Mg^{2+}). Xenografts consisting of 10 million 9L cells were injected subcutaneously (0.2 mL) into the right shoulder flank of the Fischer 344 rat. Rats were monitored on a daily basis. Proliferation of tumor took 12 - 15 days to reach appropriate size of 1 cm^3. Six rats per group were used for the biodistribution study. All animals were fasted for 12 - 18 hours prior to the study. Rats were then put under anesthesia with the use of isoflurane (2 - 3%) and 0.20 mL saline solution containing 925 KBq of [^{18}F](2S,4R)4-fluoroglutamine was injected IV. The rats were sacrificed at 30 and 60 minutes post-injection by cardiac excision while under isoflurane anesthesia. The organs of interest were removed, weighed and the radioactivity was counted with a gamma counter (Packard Cobra). The percent dose per gram was calculated by a method described above.

Supplemental figure 1: Uptake of [^{18}F](*2S,4R*)4-fluoroglutamine in 9L cells in presence of inhibitors for system A, ASC, L and N (A), in presence of specific inhibitor for ASCT2 (B) and in Na^+ free media and at different pH (C). Values are represented as mean ± SD, n = 3.



References in the supplemental section:

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