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Preloading with L-BPA, L-tyrosine and L-DOPA enhances the uptake of [^{18}F]FBPA in human and mouse tumour cell lines

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HIGHLIGHTS

- Uptake of [^{18}F]FBPA was assessed in HuH-7, CaCo-2 and B16-F1 cells.
- [^{18}F]FBPA uptake is highly dependent on the incubation medium.
- [^{18}F]FBPA uptake was higher after preloading with L-BPA, L-DOPA and L-tyrosine.

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ABSTRACT

Aim of this study was to investigate if cellular [^{18}F]FBPA uptake can be increased upon preloading with amino acids. [^{18}F]FBPA uptake was assessed in HuH-7, CaCo-2 and B16-F1 cells pretreated with different concentrations or incubation times of L-BPA, L-tyrosine or L-DOPA. Without preloading, highest uptake of [^{18}F]FBPA was observed in B16-F1 cells, followed by CaCo-2 cells and HuH-7 cells. In all cell lines higher [^{18}F]FBPA accumulation (up to 1.65-fold) was obtained with increasing L-BPA, L-DOPA and L-tyrosine concentrations.

1. Background

Boron neutron capture therapy (BNCT) is a biologically and physically targeted therapy based on the nuclear capture and fission reaction that occurs when non-radioactive ^{10}B is irradiated with low-energy thermal neutrons, creating high linear energy transfer (LET) alpha (^{4}He) and lithium (^{7}Li) particles. The average path of the secondary particles thus produced is shorter than the diameter of tumour cells, thereby damaging a well-localized area and leading to a selective, complete ablation of the malignant tissue (Barth et al., 2005). The success of BNCT requires a high accumulation of ^{10}B in the tumour cells. In the 1960 s a wide variety of boron carriers were synthesized to selectively deliver ^{10}B atoms to tumour cells. Although international efforts are devoted to synthesis of new boron carriers to enhance ^{10}B concentration in tumour cells, only two compounds, namely 4-borono-L-phenylalanine (L-BPA) and sodium borocaptate (BSH), are authorised for use in clinical trials (Henriksson et al., 2008; Kankaanranta et al., 2011; Mishima et al., 1989; Skold et al., 2010). L-BPA is an analogue of phenylalanine which is actively taken up by tumour cells (Nichols et al., 2002). To predict ^{10}B concentration in tumour cells the ^{18}F-labelled analogue of L-BPA, 2-[^{18}F]fluoro-4-borono-L-phenylalanine ([^{18}F]FBPA), can be used together with positron emission tomography (PET) (Hanaoka et al., 2014; Ishiwata et al., 1991; Wang et al., 2004). In several studies in patients undergoing [^{18}F]FBPA-PET and BNCT a comparable tumour distribution of [^{18}F]FBPA and L-BPA was found (Evangelista et al., 2013; Imahori et al., 1998; Menichetti et al., 2009; Nariai et al., 2009).

However, L-BPA and [^{18}F]FBPA not only accumulate at high concentrations in tumour cells but also in normal tissue, which limits the efficacy of BNCT (Coderre et al., 1987). A more selective and higher accumulation of ^{10}B in tumour cells would therefore be desirable, as this could increase the efficacy and reduce the

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toxicity of BNCT. One promising method for increasing the accumulation of L-BPA in tumour cells is pretreatment with compounds targeting the L or A amino acid transport systems. It was shown that [^{18}F]FBPA and L-BPA are taken up into cells by system L, which recognizes neutral amino acids with large, branched, or aromatic side chains such as phenylalanine. Of the four isoforms LAT1 (SLC7A5), LAT2 (SLC7A8), LAT3 (SLC43A1) and LAT4 (SLC43A2), LAT1 is the most important transporter as many tumours exhibit an overexpression of LAT1 (Babu et al., 2003; Bodoy et al., 2005; del Amo et al., 2008; Yoshimoto et al., 2013). LAT1 and LAT2 are amino acid exchangers with 1:1 stoichiometry, and the activity of transport depends on the availability of intracellular substrate amino acids (*trans*-stimulation) (Shotwell et al., 1983). This means that net transport of a given amino acid can only be achieved in exchange for other amino acids that might have been taken up by another (unidirectional) amino acid transporter (Lahoutte et al., 2004). It has been shown that preloading with L type amino acids enhances accumulation of L-BPA in tumour cells. L-tyrosine and L-DOPA have been described as enhancers of L-BPA accumulation in malignant glioma and melanoma cells (Capuani et al., 2008; Capuani et al., 2009; Papaspyrou et al., 1994; Wittig et al., 2000; Yang et al., 2014). However, to the best of our knowledge no study has so far evaluated the effect of amino acid preloading on [^{18}F]FBPA tumour cell uptake. The rationale for investigating the effect of preloading on [^{18}F]FBPA tumour uptake is to obtain a pharmacokinetic model that can then be applied to predict L-BPA tumour uptake.

The aim of the present study was to investigate the influence of L-BPA, L-tyrosine and L-DOPA preloading on the cellular uptake of [^{18}F]FBPA in human hepatocellular carcinoma, human colorectal adenocarcinoma cells and mouse melanoma cells. This in vitro study is the basis for subsequent preclinical and clinical studies targeting primary and secondary liver cancer in which we want to provide evidence that [^{18}F]FBPA-PET can be used for selecting liver cancer patients who may benefit from extracorporal liver BNCT (Wittig et al., 2008; Zonta et al., 2009; Zonta et al., 2006).

2. Materials and methods

2.1. Cell culture

The human hepatocellular carcinoma cell line HuH-7 was purchased from the "Institut für angewandte Zellkultur" (IAZ, Munich, Germany). The human colorectal adenocarcinoma cell line CaCo-2 (ATCC HTB-37) and the mouse melanoma cell line B16-F1 (ATCC CRL-6323) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HuH-7 cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM, Gibco by Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco). CaCo-2 cells were cultured in Minimal Essential Medium (MEM, Gibco) supplemented with 20% FBS, 1 mM non-essential amino acids (NEAA, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. B16-F1 cells were maintained in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. All cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.2. Synthesis of [^{18}F]FBPA

[^{18}F]FBPA was synthesized with a specific activity of 257 ± 37 MBq/μmol (n=22) and a radiochemical purity >98% in a TRACERlab™ FX_{FDG} synthesis module (General Electric Healthcare, Uppsala, Sweden) as described previously (Mairinger et al., 2015).

2.3. In vitro uptake of [^{18}F]FBPA

Tumour cells (10^5 cells/well) were seeded in 24-well plates, allowed to attach to the well ground and grown for 48 h. After removing the culture medium, cells were rinsed with warm Hank's Balanced Salt Solution (HBSS, Gibco) and incubated with either medium (DMEM, MEM) or HBSS containing [^{18}F]FBPA (370 kBq per well) for 15, 30, 60, 120 or 240 min at 37 °C. Then, cells were washed twice with ice-cold HBSS and detached by adding 100 μl NP40 Cell Lysis Buffer (Novex by Life Technologies, Carlsbad, CA, USA). Radioactivity retained in the lysed cells was measured using a gamma counter (Wizard 1470, PerkinElmer, Wellesley, MA) and expressed as percentage of the amount of total applied radioactivity (AR) used in the assay per 10^5 cells (%AR/10^5 cells). Data are given as mean ± standard deviation over the indicated number of experiments.

2.4. Preloading with L-BPA, L-tyrosine or L-DOPA

The effect of preloading with variable concentrations or variable incubation times was assessed in HuH-7, CaCo-2 and B16-F1 cells. In a first series of experiments, cells were incubated with 0.1, 1, 2.5 or 5 mM L-BPA, L-tyrosine or L-DOPA for 60 min at 37 °C (n=9 per concentration for HuH-7 and CaCo-2 cells, n=6 per concentration for B16-F1 cells). In a second series of experiments, cells were incubated with 2.5 mM L-BPA, L-tyrosine or L-DOPA (all obtained from Sigma Aldrich) for 15, 30, 60 or 90 min at 37 °C (n=6 for HuH-7 and CaCo-2 cells, n=3 for B16-F1 cells). After removal of the supernatant, cells were washed with warm HBSS and incubated with HBSS containing [^{18}F]FBPA for 60 min. Then cells were washed twice with ice-cold HBSS and detached by adding 100 μl NP40 Cell Lysis Buffer. Radioactivity in the lysed cells was measured in a gamma counter and expressed as %AR/10^5 cells. In addition, the relative uptake ratio as compared to untreated control (fold untreated control) was calculated for all the preloading experiments. Data are given as mean ± standard deviation over the indicated number of experiments.

2.5. Cell viability assay

Cell viability was tested by the WST-1 assay (Roche Applied Science, Mannheim, Germany). Briefly, cells were cultured as described in Section 2.3. After removing cell culture medium, cells were incubated with 5 mM L-BPA, L-tyrosine or L-DOPA for 90 min. After removal of the supernatant, cells were washed with warm HBSS and incubated with HBSS containing [^{18}F]FBPA for 60 min. Thereafter, cells were washed twice with ice-cold HBSS and incubated with WST-1 reagent (1/10th of the culture volume) for 1 h at 37 °C. The formazan dye formed was quantified by measuring the absorption at 450 nm using a BioTek ELX800 absorbance reader (BioTek, VT, USA). Viability was expressed as percent of viable cells, calculated from the ratio of absorption after pretreatment to mean absorption for the untreated control. A viability lower than 75% would be regarded as a cytotoxic effect on the cells.

2.6. Immunoblotting

Whole cell extracts of HuH-7, CaCo-2 and B16-F1 cells were prepared for detection of LAT1 (SLC7A5) expression. Briefly, cells were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). The supernatant was discarded and the remaining pellet was resuspended in ice-cold NP40 cell lysis buffer. After homogenization and centrifugation (10 min,