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A comparative small-animal PET evaluation of [¹¹C]tariquidar, [¹¹C]elacridar and (*R*)-[¹¹C]verapamil for detection of P-glycoprotein expressing murine breast cancer

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Abstract

Purpose—One important mechanism for chemoresistance of tumours is overexpression of the adenosine triphosphate-binding cassette transporter P-glycoprotein (Pgp). Pgp reduces intracellular concentrations of chemotherapeutic drugs. Aim of this study was to compare the suitability of the radiolabelled Pgp inhibitors [¹¹C]tariquidar and [¹¹C]elacridar with the Pgp substrate radiotracer (*R*)-[¹¹C]verapamil to discriminate tumours expressing low and high levels of Pgp using small-animal PET imaging in a murine breast cancer model.

Methods—Murine mammary carcinoma cells (EMT6) were continuously exposed to doxorubicin to generate a Pgp overexpressing, doxorubicin-resistant cell line (EMT6AR1.0 cells). Both cell lines were subcutaneously injected in female athymic nude mice. One week after implantation, animals underwent PET scans with [¹¹C]tariquidar (n=7), [¹¹C]elacridar (n=6) and (*R*)-[¹¹C]verapamil (n=7), before and after administration of unlabelled tariquidar (15 mg/kg). Pgp expression in tumour grafts was studied by Western blotting.

Results—[¹¹C]Tariquidar showed significantly higher retention in Pgp overexpressing EMT6AR1.0 compared with EMT6 tumours (mean area under the time-activity curve in scan 1 from time 0 to 60 min, AUC₀₋₆₀±SD: 38.8±2.2 min vs. 25.0±5.3 min, p=0.016, Wilcoxon matched pairs test). [¹¹C]Elacridar and (*R*)-[¹¹C]verapamil were not able to discriminate Pgp expression in tumour models. Following administration of unlabelled tariquidar, both EMT6Ar1.0 and EMT6 tumours showed increases in tumoural uptake of [¹¹C]tariquidar, [¹¹C]elacridar and (*R*)-[¹¹C]verapamil.

Conclusions—Among the tested radiotracers, [¹¹C]tariquidar performed best in discriminating high from low Pgp expressing tumours. Therefore [¹¹C]tariquidar merits further investigation as a PET tracer to assess Pgp expression levels of solid tumours.

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Conflicts of interest The authors declare that they have no conflict of interest.

Keywords

Multidrug resistance; P-glycoprotein; positron emission tomography; [^{11}C]tariquidar; [^{11}C]elacridar; (*R*)-[^{11}C]verapamil

Introduction

Multidrug resistance (MDR) remains a major challenge for successful systemic treatment of cancer. MDR may either be intrinsic or acquired and can be mediated by several different mechanisms including target alterations, enhanced DNA repair, evasion of apoptosis, induction of drug-metabolising enzymes, alterations in drug uptake and active transport of drugs out of cells [1]. Energy-dependent efflux of chemotherapeutic drugs out of cells is to a great extent mediated by transmembrane transporters belonging to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, which use ATP-hydrolysis as energy source to transport a variety of structurally diverse molecules across membranes irrespective of concentration gradient [2].

Among the 48 members of the human ABC transporter family, P-glycoprotein (Pgp, ABCB1) is the most widely studied one. A multitude of studies have linked increased expression of Pgp to impaired chemotherapy response and lower patient survival [3]. For over two decades, different strategies to inhibit Pgp and to overcome MDR in cancer have been investigated but were largely unsuccessful due to several different reasons [1, 4-5]. First and second generation Pgp inhibitors were either toxic at concentrations needed to inhibit Pgp (verapamil, cyclosporine A, quinidine) or prone to drug-drug interactions involving cytochrome P450 inhibition (valsopodar, biricodar) leading to increased systemic exposure and hence toxicity of chemotherapeutic drugs. Third generation Pgp inhibitors, such as tariquidar, elacridar, zosuquidar and laniquidar, were more potent and selective than earlier-generation Pgp inhibitors and lacked pharmacokinetic interactions, but still caused toxicities in cancer patients and failed to improve patient survival which resulted in early closure of several clinical trials [4-5].

A substantial limitation of most clinical trials with Pgp inhibitors is that Pgp expression in tumours of study participants was not assessed, which is not surprising given the invasive nature of biopsies and the lack of alternative methodology. To improve future clinical trials with Pgp inhibitors, a non-invasive molecular imaging protocol, which is able to assess Pgp expression and/or function, might be very useful in selecting patients whose cancers are likely to be chemoresistant through a Pgp-mediated mechanism [6]. One of the most widely used imaging agents to assess Pgp function is the single-photon emission computed tomography (SPECT) tracer [$^{99\text{m}}\text{Tc}$]sestamibi, which is a substrate of both Pgp and multidrug resistance protein 1 (MRP1, ABCC1) [7]. In some clinical trials with Pgp inhibitors, imaging of [$^{99\text{m}}\text{Tc}$]sestamibi uptake has been used as a surrogate marker for tumoural Pgp inhibition [8-10]. However, [$^{99\text{m}}\text{Tc}$]sestamibi is not an ideal Pgp imaging agent as it is not selective for Pgp and shows very low retention in Pgp expressing tumours due to rapid washout [7].

An imaging probe for the highly sensitive and quantitative imaging method positron emission tomography (PET) would be preferred over a SPECT imaging agent to accurately and non-invasively measure Pgp in tumours. Some PET tracers for Pgp including 4-[^{18}F]fluoropaclitaxel [11], [^{11}C]verapamil [12] and [^{11}C]daunorubicin [12] have been developed and evaluated in various tumour xenograft animal models. However, all these PET tracers are substrates of Pgp and are therefore efficiently kept out from the tumour by Pgp transport leading to very low imaging signals in high Pgp expressing tumours.

We have recently developed [^{11}C]tariquidar and [^{11}C]elacridar as PET tracers to visualise Pgp expression levels rather than Pgp function [13-14]. These radiotracers were shown to be hardly metabolised in mice and rats during the time course of a PET scan. An initial preclinical evaluation showed that [^{11}C]tariquidar and [^{11}C]elacridar were not able to visualise Pgp expression levels at the rodent blood-brain barrier (BBB) which could be most likely attributed to the low density of Pgp at the BBB [13-14].

Aim of this study was to assess the suitability of [^{11}C]tariquidar and [^{11}C]elacridar to visualise Pgp expression levels in a murine breast cancer model of doxorubicin-resistant and doxorubicin-sensitive tumours. For comparison, the Pgp substrate (*R*)-[^{11}C]verapamil [15-17] was evaluated as reference radiotracer in the same model.

Material and methods

Material

All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany) or Merck (Darmstadt, Germany) at analytical grade and used without further purification. Waymouth's MB 752/1, RPMI and heat inactivated fetal bovine serum (FBS) were purchased from GIBCO® (Invitrogen, Lofer, Austria). Penicillin/streptomycin solution was purchased from PAA Laboratories (Pasching, Austria). Doxorubicin hydrochloride was dissolved in sterile water at a concentration of 5 mg/ml, stored as aliquots at -30°C and diluted immediately prior to use. PhosSTOP tablets and cOmplete Protease Inhibitor cocktail were obtained from Roche Diagnostics (Mannheim, Germany). The anti-Pgp primary antibodies C219 and H241 were obtained from Signet™ (Dedham, MA, USA) or from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), respectively. The breast cancer resistance protein (BCRP, ABCG2) primary antibody BXP-53, which recognises an internal epitope in murine Bcrp1 and human BCRP, was purchased from Abcam (Cambridge, UK). Anti-actin primary antibody was purchased from Sigma (Saint Louis, MO, USA). Secondary antibodies were purchased either from Santa Cruz Biotechnology Inc. or from Jackson ImmunoResearch Inc. (West Grove, PA, USA). Antibody diluent and serum-free Protein Block solution were purchased from Dako (Glostrup, Denmark). Isoflurane was obtained from Baxter Vertriebs GmbH (Vienna, Austria).

Tariquidar dimesylate and elacridar hydrochloride were synthesised at the Department of Medicinal Chemistry (University of Vienna, Austria). Tariquidar dimesylate was freshly dissolved in 2.5% (w/v) aqueous (aq.) dextrose solution prior to each administration and injected into animals at a volume of 4 ml/kg. For *in vitro* autoradiography, elacridar hydrochloride and racemic verapamil hydrochloride were dissolved in 20% (v/v) aq. ethanol solution immediately prior use.

Cell culture

Mouse mammary carcinoma cells EMT6 (CRL-2755) and mouse mammary gland adenocarcinoma cells JC (CRL-2116) were obtained from the American Type Culture Collection (ATCC, Virginia, USA). Drug resistant human ovarian NCI/ADR-Res cells were purchased from the National Cancer Institute (Frederick, MD, USA). The BCRP overexpressing human breast adenocarcinoma cell line MCF7AdVp3000 was kindly provided by Dr. Susan E. Bates (National Cancer Institute, NIH, Bethesda, USA). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 .

EMT6 cells were cultured as monolayers in Waymouth's MB 752/1 medium supplemented with 2.4 mM of L-glutamine, 15% heat inactivated FBS, 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin. The Pgp overexpressing EMT6Ar1.0 cell line was derived by *in vitro* culture of EMT6 cells in presence of increasing concentrations of doxorubicin as

described previously [18]. JC and NCI/ADR-Res cells were cultured according to the supplier's instructions. BCRP overexpressing MCF7AdVp3000 cells were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS supplemented with 3000 ng/ml doxorubicin and 5 µg/ml verapamil.

Western blotting

Cells (2×10^5) were cultured in 6-well plates for 48 h and whole cell extracts were prepared. Snap frozen tumour grafts were pulverised with a MM200 mixer mill (Retsch, Haan, Germany) and proteins were extracted. 15 to 20 µg total protein per lane was loaded on a 10% to 13% SDS-polyacrylamide gel and blotted on a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h, followed by incubation with the primary antibodies (C219 1:100, BXP-53 1:100, anti-actin 1:5000) at 4°C overnight. Subsequently, membranes were washed and incubated with secondary antibodies (dilution 1:4000). The bound antibody was detected by chemiluminescence using enhanced luminol-based chemiluminescent reagents (Pierce, Rockford, IL, USA) and exposure of the membranes to chemiluminescence hyperfilms (GE Healthcare, Piscataway, NJ, USA). Human breast carcinoma whole cell lysate ZR-75-1 (Santa Cruz, CA, USA) was used as control to confirm the identity of the signals, JC cells were used as control for the protein extraction procedure as JC cells express Pgp *in vitro* [19]. BCRP expressing MCF7AdVp3000 cells were used as positive control for Bcrp1 [20]. Expression of β-actin was measured to confirm consistent gel loading.

Immunocytochemistry

Immunocytochemical detection of Pgp on chamber slide cultivated tumour cells was performed using rabbit polyclonal antibody H241. Experimental details are given in the Electronic Supplementary Material.

In vitro cytotoxicity assay

EMT6 and EMT6Ar1.0 cells were treated *in vitro* with increasing concentrations of tariquidar in their respective culture media and cell survival was measured. Experimental details are given in the Electronic Supplementary Material.

Animals

Female athymic nude NMRI-Foxn1^{nu} mice (Taconic, Ry, Denmark) aged 6-8 weeks, weighing 25-30 g were housed in Makrolon Type 2 filter-top cages (3-4 mice per cage) under controlled environmental conditions (24±2°C, 40-70% humidity, 12 h light/dark cycle). An acclimatisation period of >1 week was allowed before animals were used for the experiments. EMT6 and EMT6Ar1.0 cells were harvested and resuspended in PBS. $3-5 \times 10^5$ cells were subcutaneously injected in the left and right upper flank, respectively. When tumours were grown to a size of approximately 130 mm³, animals underwent PET imaging. The study was approved by the local animal welfare committee and all study procedures were performed in accordance with the Austrian Animal Experiments Act.

Tracer synthesis and formulation

[¹¹C]Tariquidar, [¹¹C]elacridar and (*R*)-[¹¹C]verapamil were synthesised as described previously [13-14, 16]. For intravenous (i.v.) injection into animals, each radiotracer was formulated in 0.9% aq. saline containing 75 µl of Tween 80 to an approximate concentration of 370 MBq/ml. Radiochemical purity, as determined by radio-HPLC, was greater than 98% and specific activity at end of synthesis was >100 GBq/µmol. [¹⁸F]FDG was obtained from Seibersdorf Labor GmbH (Seibersdorf, Austria) and diluted for i.v. injection with 0.9% aq. saline to a final concentration of approximately 74 MBq/ml.

Small-animal PET

Prior to each experiment, the animals were placed into an induction box and anaesthetised with 2.5% isoflurane. During the imaging period anaesthesia was maintained with 1-2% isoflurane administered *via* a cone mask and the isoflurane level was adjusted depending on the depth of anaesthesia. Animal respiratory rate and body temperature were constantly monitored during the data acquisition period. The animals were kept warm throughout the experiment at approximately 37°C. Mice were positioned in a custom-made imaging chamber and a lateral tail vein was cannulated for radiotracer administration.

For PET imaging a microPET Focus220 scanner (Siemens Medical Solutions, Knoxville, TN, USA) was used. Animals were divided into three groups. Animals of group 1 (n=7), group 2 (n=6) and group 3 (n=7) underwent two consecutive dynamic PET scans with [¹¹C]tariquidar (injected activity: 40±8 MBq), [¹¹C]elacridar (injected activity: 39±10 MBq), or (*R*)-[¹¹C]verapamil (injected activity: 39±4 MBq), respectively (see Fig. 2). Scan 1 (150 min), during which unlabelled tariquidar (15 mg/kg) was injected i.v. at 60 min after radiotracer injection, was followed by scan 2 (60 min), which was recorded at 120 min after unlabelled tariquidar administration [13-14]. At the end of scan 2, a 20 min static [¹⁸F]FDG scan was performed to facilitate definition of tumour regions of interest (ROI). An additional group (n=7) of animals, underwent single 10 min static PET scans at 60 min after injection of [¹⁸F]FDG (6±3 MBq).

For all groups, list-mode data were acquired for the defined time period with an energy window of 250-750 keV and a 6 ns timing window. Before each PET scan, a transmission scan using a ⁵⁷Co point source was recorded for 10 min.

After completion of the imaging procedure, animals were sacrificed by cervical dislocation while still under deep anaesthesia. Tumours were excised and either snap frozen in liquid nitrogen or embedded in freezing medium (Tissue-Tek, Sakura, Netherlands) and frozen in isopentane (-70°C). Samples were stored at -80°C until further proceeded.

In vitro autoradiography

Tumours were cut in 10 µm slices using a cryostat (Microm HM550, Walldorf, Germany) at -12°C. Consecutive tumour sections (n=3) were mounted on SuperFrost® Plus slides (Menzel, Braunschweig, Germany), air-dried and stored at -30°C. On the day of the experiment, the slides were thawed and preincubated in PBS. Subsequently, tumour slices were incubated for 30 min with either radiotracer alone (about 20 MBq) or radiotracer with unlabelled tariquidar (10 µM) or unlabelled elacridar (10 µM). In the case of (*R*)-[¹¹C]verapamil one set of slides was additionally treated with unlabelled racemic verapamil (10 µM). To remove non-specifically bound radiotracer, slides were then washed with 20% (v/v) ethanol in PBS and distilled water. The slides were air-dried and exposed to a multisensitive phosphor screen (MS, Perkin-Elmer Life Sciences, Waltham, MA, USA). The screens were removed from the film cassettes under dim light conditions and immediately scanned at 600 dpi resolution using a Cyclone storage phosphor system (Perkin-Elmer Life Sciences, Waltham, MA, USA). Images were analysed using the system's Optiquant v5.0 software. Tumours ROIs were outlined for which the background corrected digital luminescence units per mm² were calculated.

PET data analysis

The dynamic PET data were sorted into three-dimensional sinograms. Images were reconstructed using Fourier rebinning of the 3-D sinograms followed by two-dimensional filtered backprojection. The standard data correction protocol (normalisation, decay correction and injection decay correction) was applied to the data. Tumour ROIs were

manually outlined over multiple planes in the static [^{18}F]FDG scans and whole brain ROIs were outlined in the PET summation images of scan 1. ROIs were used to generate volumes of interest (VOI), which were then transferred to the PET images of the individual time frames. Time-activity curves (TAC), expressed as standardised uptake value ($\text{SUV} = (\text{radioactivity per cubic centimetre/injected radioactivity}) \times \text{body weight}$), were calculated for each VOI and areas under the TACs from 0 to 60 min after radiotracer injection (AUC_{0-60}) were calculated.

Statistical analysis

Differences between tumour and brain AUC_{0-60} values in scan 1 and scan 2 were analysed by a two-sided Wilcoxon matched pairs signed rank test using PRISM 5 software (GraphPad Software Inc., La Jolla, CA, USA). The level of statistical significance was set to $p < 0.05$.

Results

Pgp overexpression

In vitro, parental EMT6 cells showed only very low Pgp expression, whereas a dose-dependent increase in Pgp expression was observed following exposure of cells to increasing concentrations of doxorubicin (Fig. 1a). Western blotting of excised tumours (Fig. 1b) confirmed that Pgp overexpression observed in EMT6Ar1.0 cell lysates was maintained *in vivo*. However, as compared to the corresponding cell lysates (Fig. 1a), differences in Pgp expression between EMT6Ar1.0 and EMT6 tumours appeared to be smaller (Fig. 1b). Because tariquidar and elacridar are known to interact with BCRP, we also investigated Bcrp1 expression levels of cell lysates and tumours. Only basal levels of Bcrp1 expression could be detected in EMT6 cells which were not different from the doxorubicin exposed EMT6Ar1.0 cell line (Fig. 1c). Also *in vivo*, comparably low Bcrp1 expression levels were detected in EMT6 and EMT6Ar1.0 tumours (Fig. 1d). Immunocytochemical detection of Pgp is shown in Fig. S1 (Electronic Supplementary Material). In EMT6 cells only weak Pgp staining, mostly in intracellular vesicles, was observed (Fig. S1a). In EMT6Ar1.0 cells an overall much more intensive staining for Pgp was detected, mainly at the plasma membrane, with a variable degree of staining intensity between individual cells (Fig. S1c).

Pgp expression confers a chemoresistance phenotype *in vitro*

To confirm expression of functional Pgp, EMT6 and EMT6Ar1.0 cells were treated with increasing concentrations of tariquidar (0.01 to 10 μM , Fig. S2, Electronic Supplementary Material). After 48 h incubation time, addition of tariquidar dose-dependently restored the sensitivity of EMT6Ar1.0 cells to doxorubicin whereas no effect of tariquidar on cell survival was observed in EMT6 cells. Cell survival rates of EMT6Ar1.0 cells decreased to $< 5\%$ of control cells when incubated with tariquidar (0.1 μM) for 48 h.

Small-animal PET shows increased uptake of [^{11}C]tariquidar in Pgp overexpressing tumours

Two consecutive PET scans were performed with [^{11}C]tariquidar, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil, before and after administration of unlabelled tariquidar (15 mg/kg), in mice bearing EMT6 and Pgp overexpressing EMT6Ar1.0 tumours. In Figure 2, a diagram of the study set-up is shown. Figure 3 shows representative PET summation images before and 2 h after tariquidar administration for all 3 radiotracers and corresponding [^{18}F]FDG PET images, which were employed for definition of tumour ROIs. TACs of [^{11}C]tariquidar, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil are shown in Figure 4 and scatter plots of AUC_{0-60} values in individual tumours are shown in Figure 5.

In scan 1, uptake of [^{11}C]tariquidar (Fig. 3-4a) and [^{11}C]elacridar (Fig. 3-4b) was higher in EMT6Ar1.0 than in EMT6 tumours. In contrast, (*R*)-[^{11}C]verapamil uptake was lower in EMT6Ar1.0 than in EMT6 tumours (Fig. 3-4c). For [^{11}C]tariquidar, mean AUC_{0-60} values ($\pm$ standard deviation, SD) in scan 1 were 38.8 ± 2.2 min in EMT6Ar1.0 and 25.0 ± 5.3 min in EMT6 tumours ($p=0.016$) (Fig. 5a). For [^{11}C]elacridar (Fig. 5b) and (*R*)-[^{11}C]verapamil (Fig. 5c) differences in AUC_{0-60} between tumours were not statistically significant. In response to tariquidar administration during scan 1, TACs of all 3 radiotracers showed modest increases both in EMT6Ar1.0 and EMT6 tumors (Fig. 4). In brain, which was assessed as a reference region for Pgp modulation at the BBB, tariquidar-induced increases in activity uptake during scan 1 were more pronounced than in tumors for all 3 radiotracers (Fig. 4, 5). For all radiotracers, tumoral activity levels were 2-3 times higher than brain activity levels in scan 1.

In scan 2, which was recorded at 2 h after tariquidar administration, tumoural AUC_{0-60} values were increased (range: 9-30%) relative to scan 1 both in EMT6Ar1.0 and EMT6 tumours for all 3 radiotracers, although none of these increases reached statistical significance (Fig. 5). Moreover, AUC_{0-60} increases in scan 2 were not significantly different between EMT6Ar1.0 and EMT6 tumours for all radiotracers. As for scan 1, [^{11}C]tariquidar was the only radiotracer in scan 2 where significant differences between AUC_{0-60} in EMT6Ar1.0 and EMT6 tumours were detected (mean $\text{AUC}_{0-60} \pm \text{SD}$: 45.6 ± 4.8 vs. 31.7 ± 7.9 min, $p=0.016$) (Fig. 5a).

In brain, AUC_{0-60} values in scan 2 were 2.4 ± 0.3 ($p=0.016$), 3.5 ± 0.4 ($p=0.031$) and 3.3 ± 0.5 times ($p=0.031$) increased relative to scan 1 for [^{11}C]tariquidar, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil, respectively (Fig. 5). For [^{11}C]elacridar and (*R*)-[^{11}C]verapamil, but not for [^{11}C]tariquidar, activity levels in brain were higher in scan 2 than tumoural activity levels.

In Figure S3 (Electronic Supplementary Material), a representative, static [^{18}F]FDG PET image and scatter plots of [^{18}F]FDG SUVs in EMT6 and EMT6Ar1.0 tumours obtained in a separate group of animals are shown. Tumours were clearly visible in the [^{18}F]FDG PET images indicating functional tumour metabolism and vascularisation. Tumoural SUVs were significantly higher in EMT6Ar1.0 than in EMT6 tumours (mean $\text{SUV} \pm \text{SD}$: 2.45 ± 0.75 vs. 1.86 ± 0.49 , $p=0.016$).

***In vitro* autoradiography shows higher binding of [^{11}C]tariquidar and [^{11}C]elacridar in Pgp overexpressing tumours**

Figure 6 shows images from *in vitro* autoradiography performed with [^{11}C]tariquidar and [^{11}C]elacridar on EMT6 and EMT6Ar1.0 tumour sections. In contrast to EMT6 tumours, EMT6Ar1.0 tumours displayed dense [^{11}C]tariquidar and [^{11}C]elacridar binding. Incubation of both tumour types with (*R*)-[^{11}C]verapamil yielded very noisy images with weak image contrast (images not shown). Quantitative image analysis showed 4.3 and 2.1 times higher binding of [^{11}C]tariquidar (Fig. 6b) and [^{11}C]elacridar (Fig. 6d) in EMT6Ar1.0 tumours compared to EMT6 tumours, respectively. Co-incubation with unlabelled tariquidar ($10 \mu\text{M}$) or elacridar ($10 \mu\text{M}$) reduced [^{11}C]tariquidar and [^{11}C]elacridar binding in EMT6Ar1.0 tumours to similar levels as in EMT6 tumours (Fig. 6b,d). Binding of (*R*)-[^{11}C]verapamil in EMT6Ar1.0 tumours was only 1.3 times higher than in EMT6 tumours with no significant reduction in both tumours after co-incubation with unlabelled tariquidar, elacridar or verapamil (Fig. 6e).

Discussion

Aim of this study was to compare the suitability of [^{11}C]tariquidar and [^{11}C]elacridar with (*R*)-[^{11}C]verapamil, a commonly used radiotracer to assess Pgp function [15-17], to

discriminate Pgp positive from Pgp negative tumours using small-animal PET. We show that [^{11}C]tariquidar displays significantly higher *in vivo* binding in the Pgp overexpressing EMT6Ar1.0 murine breast cancer model as compared to EMT6 tumours, which have only low Pgp expression. In contrast, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil were not able to discriminate the two different tumour models.

Tariquidar and elacridar are potent non-competitive inhibitors of Pgp, which inhibit ATPase activity, suggesting that the modulating effect is derived from the inhibition of substrate binding, inhibition of ATP hydrolysis or both [4]. [^{11}C]Tariquidar and [^{11}C]elacridar were developed as PET tracers to visualise Pgp expression levels at the BBB [13-14, 21-22], as opposed to radiolabelled Pgp substrates, such as (*R*)-[^{11}C]verapamil, which visualise Pgp function [15-17]. Even though Pgp expression levels might not necessarily correlate with Pgp functionality such probes are expected to give higher signals than radiolabelled Pgp substrates potentially making them more sensitive to detect Pgp *in vivo*. However, there is evidence that [^{11}C]tariquidar and [^{11}C]elacridar may be transported by Pgp and/or BCRP at the rodent BBB. This resulted in low baseline brain uptake which was several times increased following pre-treatment of animals with unlabelled tariquidar or elacridar [13-14, 21-22]. We speculate that the density of Pgp at the rodent BBB is too low in relation to the Pgp binding affinities of tariquidar and elacridar ($K_d = 5.1$ nM for binding of [^3H]tariquidar to plasma membrane preparations of Pgp overexpressing CH'B30 cells [23]) to provide a Pgp specific binding signal with these radiotracers.

In the present study, tumoural activity uptake of [^{11}C]tariquidar and [^{11}C]elacridar was shown to be 2 to 3 times higher than activity uptake in brain suggesting better suitability of these radiotracers for tumour than for brain imaging. An advantage of the employed model was that doxorubicin-sensitive and resistant tumours were derived from the same cell line and implanted bilaterally into animals so that each animal could serve as its own control. A possible limitation of the employed model may be that tumours were derived from a murine and not from a human cell line, which could limit the extrapolation of the results to human tumours. Moreover, it cannot be excluded that Pgp expression levels of murine EMT6Ar1.0 tumours were different (i.e. higher) from those in clinical tumour samples. [^{11}C]Tariquidar was found to display 50% higher retention in Pgp overexpressing EMT6Ar1.0 tumours than in EMT6 tumours, which had only low Pgp expression levels, which suggested *in vivo* binding of [^{11}C]tariquidar to Pgp (Fig. 5a). The Pgp substrate (*R*)-[^{11}C]verapamil, on the other hand, showed - although not statistically significant - lower retention in EMT6Ar1.0 than in EMT6 tumours (Fig. 5c), which pointed to Pgp-mediated efflux transport of (*R*)-[^{11}C]verapamil. Similar results have been obtained earlier with racemic (*R*)-[^{11}C]verapamil in rats xenografted with a Pgp-negative small cell lung carcinoma (GLC₄) and its Pgp-overexpressing subline (GLC₄/Pgp) [12].

Because [^{18}F]FDG PET images, which were acquired at the end of the imaging session for definition of tumoural ROIs, had pointed to higher [^{18}F]FDG retention in EMT6Ar1.0 than in EMT6 tumours (Fig. 3), we studied tumoural [^{18}F]FDG uptake in an additional group of animals, in which significantly higher [^{18}F]FDG uptake in EMT6Ar1.0 tumours was confirmed (Fig. S3). Similar results have been obtained in a previous study [24] and may be explained by higher glucose consumption due to increased basal ATPase activity or increased blood flow or anaerobic metabolism in EMT6Ar1.0 tumours.

To assess Pgp specificity of PET signal we performed PET scans in mice before, during and after administration of unlabelled tariquidar (Fig. 2), an approach already used in previous studies for assessment of Pgp specific binding of [^{11}C]tariquidar and [^{11}C]elacridar at the rodent BBB [13-14]. The employed tariquidar dose (15 mg/kg) was reported to result in complete inhibition of Pgp at the BBB [17]. Due to the long terminal elimination half-life of

tariquidar (26 h) [4] it can be expected that plasma levels of tariquidar were stable during scan 2. We observed changes in tumoural PET signal following unlabelled tariquidar administration (Fig. 5). However, PET signal was increased rather than decreased as we would have expected from displacement of tumoural Pgp binding of [^{11}C]tariquidar and [^{11}C]elacridar. Moreover, changes in PET signal following unlabelled tariquidar administration were irrespective of Pgp expression levels of tumours (Fig. 5). To elucidate, we performed *in vitro* autoradiography with [^{11}C]tariquidar, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil in EMT6Ar1.0 and EMT6 tumours (Fig. 6). Similar to the *in vivo* PET results, *in vitro* binding of [^{11}C]tariquidar and [^{11}C]elacridar was higher in Pgp overexpressing EMT6Ar1.0 than in EMT6 tumours whereas no differences in binding could be detected for (*R*)-[^{11}C]verapamil (Fig. 6). In contrast to the PET data, [^{11}C]tariquidar and [^{11}C]elacridar binding was reduced *in vitro* following co-incubation with tariquidar or elacridar (10 μM). Moreover, reduction in [^{11}C]tariquidar and [^{11}C]elacridar *in vitro* binding was greater in EMT6Ar1.0 than in EMT6 tumours (Fig. 6b,d). These apparently contradicting observations might be explained by additional, competing transport mechanisms only present in the *in vivo* model such as inhibition of efflux transport of [^{11}C]tariquidar and [^{11}C]elacridar *in vivo*. Kannan and co-workers have recently shown *in vitro* that [^3H]tariquidar is an avid BCRP substrate [25]. In line, our own results obtained in wild-type, *Mdr1a/b*^(-/-), *Bcrp1*^(-/-) and *Mdr1a/b*^(-/-)*Bcrp1*^(-/-) mice suggested that [^{11}C]tariquidar and [^{11}C]elacridar act as dual Pgp/BCRP substrates at the BBB [13-14]. *In vivo*, [^{11}C]tariquidar and [^{11}C]elacridar might both bind to Pgp and be transported by Pgp and/or BCRP, probably *via* different binding sites. It might be possible that administration of unlabelled tariquidar displaced binding of [^{11}C]tariquidar and [^{11}C]elacridar from their Pgp binding sites and at the same time increased tumoural cellular uptake by inhibition of Pgp/BCRP-mediated efflux transport of radiotracer. In line with this assumption Western blot analysis had shown that both EMT6Ar1.0 and EMT6 tumours had basal *Bcrp1* expression levels (Fig. 1c,d). In contrast to tumours, the substrate properties of [^{11}C]tariquidar and [^{11}C]elacridar seem to prevail at the BBB with its lower Pgp expression, resulting in more pronounced increases in activity uptake following tariquidar challenge in brain than in tumours (Fig. 4). A third mechanism which might play a role in tumoural retention of [^{11}C]tariquidar and [^{11}C]elacridar is intracellular trapping in lysosomes, which might be displaceable by unlabelled tariquidar [26].

We and others have shown previously that [^{11}C]tariquidar and [^{11}C]elacridar are hardly metabolised *in vivo* [13-14, 21-22] suggesting that the PET signal measured *in vivo* in tumours was due to unmetabolised radiotracer. This might pose an advantage over radiotracers such as (*R*)-[^{11}C]verapamil [15] or 4-[^{18}F]fluoropaclitaxel [11], which are extensively metabolised *in vivo*, possibly resulting in tumoural accumulation of radiolabelled metabolites.

Conclusion

Even though tumoural retention mechanisms of [^{11}C]tariquidar appeared to be complex, [^{11}C]tariquidar merits further investigation as a PET tracer to assess Pgp expression levels of solid tumours. As tariquidar is one of a few Pgp inhibitors which is still tested in clinical trials [4], use of [^{11}C]tariquidar PET to preselect patients with high Pgp expression levels for co-treatment with tariquidar is a promising personalised medicine approach in cancer therapy. Besides its potential utility as a PET tracer, radiolabelled tariquidar might be useful to quantify Pgp expression levels *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Western blot analysis of Pgp (170 kDa) and Bcrp1 (72 kDa) in parental and doxorubicin exposed (0.2, 0.4 and 1 μ g/ml) mouse mammary carcinoma (EMT6) cell lysates (**a, c**) and EMT6 and EMT6Ar1.0 derived tumours (**b, d**). Expression of Pgp and Bcrp1 was assessed using the monoclonal antibodies C219 and BXP-53, respectively. Human breast carcinoma whole cell lysate ZR-75-1 and mouse mammary gland adenocarcinoma cells (JC) were used as positive controls for Pgp, human BCRP overexpressing cells (MCF7AdVp3000) were used as positive control for Bcrp1. Equal protein loading was confirmed by measurement of β -actin expression. An unspecific band could be detected when the BXP-53 antibody was used for Bcrp1 detection

Fig. 2.

Diagram of study set-up for [^{11}C]tariquidar, [^{11}C]elacridar and (*R*)-[^{11}C]verapamil. Scan 1 (150 min), during which unlabelled tariquidar (15 mg/kg) was injected over approximately 60 s at 60 min after radiotracer injection, was followed by scan 2 (60 min), which was recorded at 120 min after unlabelled tariquidar administration. At the end of scan 2, a 20-min static [^{18}F]FDG PET scan was performed to facilitate definition of tumour ROIs

Fig. 3.

Representative, transversal PET summation images of tumour bearing mice from 30-60 min after injection of [^{11}C]tariquidar (**a**), [^{11}C]elacridar (**b**) and (*R*)-[^{11}C]verapamil (**c**) before (left column) and after (middle column) treatment with 15 mg/kg of unlabelled tariquidar. Corresponding [^{18}F]FDG images for tumour definition are shown in the right column. Radiation scale is expressed as SUV (T, tumour; H, heart; L, lungs)

Fig. 4. Mean TACs (SUV ± SD) in EMT6 (blue squares) and EMT6Ar1.0 (red squares) tumours for PET scans (left column: scan 1, right column: scan 2) with $[^{11}\text{C}]$ tariquidar (a, n=7), $[^{11}\text{C}]$ elacridar (b, n=6) and (R)- $[^{11}\text{C}]$ verapamil (c, n=7). Whole brain (black open circles) was assessed as a reference region for Pgp modulation by unlabelled tariquidar at the BBB. Tariquidar (15 mg/kg) was administered as an i.v. bolus at 60 min after start of scan 1 (arrow)

Fig. 5. AUC₀₋₆₀ values in individual EMT6 and EMT6Ar1.0 tumours and in whole brain measured with PET from 0-60 min after injection of [¹¹C]tarividar (**a**, n=7), [¹¹C]elacridar (**b**, n=6) and (R)-[¹¹C]verapamil (**c**, n=7), before (left) and after (right) unlabelled tarividar administration. Horizontal lines represent geometric means ± SD. Statistically significant differences (p < 0.05, Wilcoxon matched pairs signed rank test) are indicated by an asterisk

Fig. 6. Autoradiography images obtained after incubation of EMT6 and EMT6Ar1.0 tumour slices with [¹¹C]tariquidar (**a**) or [¹¹C]elacridar (**c**) alone (upper row) and with co-incubation with 10 µM tariquidar (middle row) or 10 µM elacridar (bottom row). Quantitative assessment of the autoradiography images (left graph: EMT6 tumour; right graph: EMT6Ar1.0 tumour) in mean net digital luminescence units/mm² tumour tissue ± SD (n=3) for [¹¹C]tariquidar (**b**), [¹¹C]elacridar (**d**) and (*R*)-[¹¹C]verapamil (**e**)