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Synthesis and preclinical evaluation of the radiolabeled P-glycoprotein inhibitor [^{11}C]MC113

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Abstract

Objectives—With the aim to develop a PET tracer to visualize P-glycoprotein (Pgp) expression levels in different organs, the Pgp inhibitor MC113 was labeled with ^{11}C and evaluated using small-animal PET.

Methods—[^{11}C]MC113 was synthesized by reaction of *O*-desmethyl MC113 with [^{11}C]methyl triflate. Small-animal PET was performed with [^{11}C]MC113 in FVB wild-type and *Mdr1a/b*^(-/-) mice ($n=3$ per group) and in a mouse model of high (EMT6Ar1.0) and low (EMT6) Pgp expressing tumor grafts ($n=5$). In the tumor model, PET scans were performed before and after administration of the reference Pgp inhibitor tariquidar (15 mg/kg).

Results—Brain uptake of [^{11}C]MC113, expressed as area under the time-activity curve from time 0 to 60 min (AUC_{0-60}), was moderately but not significantly increased in *Mdr1a/b*^(-/-) compared with wild-type mice (mean $\pm$ SD AUC_{0-60} , *Mdr1a/b*^(-/-): 88 ± 7 min, wild-type: 62 ± 6 min, $P=0.100$, Mann Whitney test). In the tumor model, AUC_{0-60} values were not significantly different between EMT6Ar1.0 and EMT6 tumors. Neither in brain nor in tumors was activity concentration significantly changed in response to tariquidar administration. Half-maximum effect concentrations (IC_{50}) for inhibition of Pgp-mediated rhodamine 123 efflux from CCRFvcr1000 cells were 375 ± 60 nM for MC113 versus 8.5 ± 2.5 nM for tariquidar.

Conclusion—[^{11}C]MC113 showed higher brain uptake in mice than previously described Pgp PET tracers, suggesting that [^{11}C]MC113 was only to a low extent effluxed by Pgp. However, [^{11}C]MC113 was found unsuitable to visualize Pgp expression levels presumably due to insufficiently high Pgp binding affinity of MC113 in relation to Pgp densities in brain and tumors.

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Keywords

P-glycoprotein; blood-brain barrier; tumor; tariquidar; MC113; MC18; positron emission tomography

1. Introduction

The adenosine triphosphate (ATP) binding cassette (ABC) transporter P-glycoprotein (Pgp) is expressed in the luminal membrane of the small intestine and blood-brain barrier (BBB), and in the apical membranes of excretory cells such as hepatocytes and kidney proximal tubule epithelia [1]. Pgp actively transports a wide range of structurally diverse, mostly lipophilic compounds against concentration gradients and may therefore exert a significant impact on absorption, distribution and excretion of drug molecules [2]. Moreover, Pgp-mediated efflux transport of chemotherapeutic drugs out of tumor cells is an important mechanism contributing to chemoresistance of tumors [3]. Apart from its well established role in tumor resistance, changes in Pgp expression and function are also believed to occur in several neurological disorders, such as epilepsy, Alzheimer's and Parkinson's disease [1]. Positron emission tomography (PET) is a potentially powerful method to non-invasively study disease related alterations in Pgp functionality and density in different organs such as the brain provided the availability of suitable radiotracers for Pgp. Most PET tracers for Pgp investigated in humans so far are high-affinity Pgp substrates, such as racemic [^{11}C]verapamil, (*R*)-[^{11}C]verapamil or [^{11}C]-*N*-desmethyl-loperamide [4-6]. Whereas these probes were found to be suitable to measure global changes in Pgp function at the BBB after pharmacological inhibition of Pgp with inhibitors, such as tariquidar or cyclosporine A, their low brain uptake makes the assessment of more subtle alterations in Pgp function/expression as they may occur in distinct brain regions during the progression of disease very challenging [4-6].

As an alternative to radiolabeled Pgp substrates, radiolabeled Pgp inhibitors, such as [^{11}C]laniquidar [7], [^{11}C]tariquidar [8, 9], [^{11}C]elacridar [10, 11], [^{18}F]fluoroethyl-elacridar and - tariquidar [12] and 1-[^{18}F]fluoroelacridar [13], have been proposed. It was expected that such probes would bind to Pgp rather than being transported by Pgp and thereby allow for mapping of Pgp density and afford higher PET signals than radiolabeled substrates. Unexpectedly, these probes were found to display very low brain uptake in rodents, most likely because they were recognized by Pgp and breast cancer resistance protein (Bcrp), another ABC transporter expressed at the BBB, as substrates [14, 15], making them unsuitable to measure Pgp density at the BBB.

Recently, a series of new potent Pgp inhibitors, which share with tariquidar and elacridar the basic 6,7-dimethoxytetrahydroisoquinoline nucleus, has been described (Fig. 1) [16]. One of these compounds, 6,7-dimethoxy-2-{3-[4-methoxy-3,4-dihydro-2H naphthalen-(1*E*)-ylidene]-propyl}-1,2,3,4-tetrahydro-isoquinoline (MC18, Fig. 1) was labeled with carbon-11 (^{11}C) and shown to display approximately four times higher brain uptake in rats than [^{11}C]tariquidar [17], suggesting that [^{11}C]MC18 is not or to a considerably lesser extent transported by Pgp and Bcrp at the BBB than [^{11}C]tariquidar. Moreover, V_T of [^{11}C]MC18 was decreased by 30% in rats pretreated with cold MC18 (15 mg/kg) pointing to some extent of Pgp-specific binding of this radiotracer [17]. The *in vivo* behavior of [^{11}C]MC18 stands in contrast with that of [^{11}C]tariquidar [8, 9], [^{11}C]elacridar [10, 11] and [^{11}C]laniquidar [18], which all showed increases in brain uptake as compared with baseline scans following pretreatment of rats or mice with the respective unlabeled compounds, presumably due to inhibition of Pgp/Bcrp efflux of these radiotracers by cold compound.

Starting from MC18 as lead, 6,7-dimethoxy-2-(4-methoxy-biphenyl-4-yl-methyl)-1,2,3,4-tetrahydro-isoquinoline (MC70, Fig. 1) has been synthesized and found to be approximately 30 times more potent than MC18 in inhibiting Pgp-mediated [^3H]vinblastine transport in Caco-2 cells [19]. Moreover, MC70 was shown to have an efflux ratio of 1.3 in transport experiments in Caco-2 monolayers, which indicated that this compound was not transported by Pgp or other transporters expressed in Caco-2 cells [19]. Based on these reported properties MC70 appears as an interesting candidate for developing a Pgp inhibitor based PET ligand to measure Pgp expression levels, which is expected to provide a higher Pgp-specific signal than [^{11}C]MC18 due to a presumably higher Pgp binding affinity.

In this work we labeled the *O*-methyl derivative of MC70, MC113 (Fig. 1), with ^{11}C . We assessed the suitability of [^{11}C]MC113 to measure Pgp expression levels *in vivo* by performing small-animal PET experiments in wild-type and Pgp knockout (*Mdr1a/b*^(-/-)) mice as well as in a recently described mouse model of high and low Pgp expressing tumor grafts [20]. Data obtained with [^{11}C]MC113 were directly compared with data which we have previously obtained with [^{11}C]tariquidar using the same *in vivo* models [9, 20].

2. Materials and methods

2.1. General

Chemicals were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany) or Merck (Darmstadt, Germany) at analytical grade and used without further purification. Tariquidar dimesylate and elacridar hydrochloride were synthesized 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 intravenously (i.v.) injected into animals at a volume of 4 mL/kg. MC113, MC70 and MC18 were synthesized at the Department of Pharmacy (University of Bari, Bari, Italy) as described previously [16, 19]. [^{11}C]Methane was produced *via* the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction by irradiation of nitrogen gas containing 10% hydrogen using a PETtrace cyclotron equipped with a methane target system (GE Healthcare, Uppsala, Sweden). [^{11}C]Methyl iodide was prepared in a TracerLab FXC Pro synthesis module (GE Healthcare) and converted into [^{11}C]methyl triflate by passage through a column containing silver-triflate impregnated graphitized carbon [21]. [^{18}F]FDG was obtained from Seibersdorf Labor GmbH (Seibersdorf, Austria).

2.2. Animals and cell lines

Female wild-type and *Mdr1a/b*^(-/-) mice with a FVB genetic background were obtained from Taconic (Germantown, USA). Female athymic nude NMRI-Foxn1^{nu} mice were obtained from Taconic (Ry, Denmark). Mouse mammary carcinoma cells EMT6 (CRL-2755) were obtained from the American Type Culture Collection (ATCC, Virginia, USA). 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 [20]. EMT6 and EMT6Ar1.0 cells were harvested and resuspended in PBS. 3 - 5 × 10⁵ cells were subcutaneously injected in the left and right upper flank of athymic nude NMRI-Foxn1^{nu} mice, respectively. When tumors were grown to a size of approximately 310±251 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. All efforts were made to minimize both the suffering and the number of animals used in this study.

2.3. Synthesis of [^{11}C]MC113

Using a TracerLab FXC Pro synthesis module [^{11}C]methyl triflate was bubbled through a solution of MC70 (free base, 0.5 mg, 1.3 μmol) in DMF (0.1 mL), acetone (0.4 mL) and aq. sodium hydroxide solution (0.3M, 3 μL). After heating for 4 min at 60°C the reaction mixture was cooled (25°C), diluted with water (0.5 mL) and injected into a built-in high-performance liquid chromatography (HPLC) system equipped with a Chromolith Performance RP-18e (100-4.6 mm) column (Merck KGaA) which was eluted with acetonitrile/methanol/water (45/10/145, v/v/v) at a flow rate of 4 mL/min. The HPLC eluate was monitored in series for radioactivity and ultraviolet (UV) absorption at a wavelength of 227 nm. On this system, radiolabeling precursor MC70 and product [^{11}C]MC113 eluted with retention times of 1.5-3 min and 7-10 min, respectively. The product fraction collected from HPLC was diluted with water (100 mL) and passed over a C18 Sep-Pak Plus cartridge (Waters, Milford, MA, USA), which had been preactivated with ethanol (5 mL) and water (10 mL). The cartridge was then washed with water (10 mL) followed by elution of [^{11}C]MC113 with ethanol (3 mL). The ethanol was then removed by heating at 90°C under a stream of argon and the product was formulated in a mixture of 0.9% aq saline/0.01 M hydrochloric acid (1/0.01, v/v) at an approximate concentration of 370 MBq/mL for i.v. injection into animals. Radiochemical purity and specific activity of [^{11}C]MC113 were determined by analytical radio-HPLC using a LiChrospher 100 RP-18e (250-4 mm 5 μm) column eluted with acetonitrile/methanol/water (350/78/572, v/v/v) at a flow rate of 1 mL/min. UV detection was performed at a wavelength of 227 nm. The retention time of [^{11}C]MC113 was approximately 6 min on this HPLC system. The identity of [^{11}C]MC113 was confirmed by HPLC co-injection with unlabeled MC113.

2.4. Experimental design for PET imaging

Two different experimental settings were used in this study. In the first set of experiments, female FVB wild-type and *Mdr1a/b*^(-/-) mice underwent single 60-min [^{11}C]MC113 PET scans ($n=3$ per group). In the second set of experiments, tumor bearing mice ($n=5$) underwent two consecutive dynamic PET scans with [^{11}C]MC113 (see Fig. 2). Scan 1 (150 min), during which cold 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 cold tariquidar administration. At the end of scan 2, a 20-min static [^{18}F]FDG scan was performed to facilitate definition of tumor regions of interest (ROI) (Fig. 2).

2.5. PET imaging

Prior to each experiment, the animals were placed in an induction box and anaesthetized with 2.5% isoflurane. During the imaging period anesthesia was maintained with 1-2% isoflurane administered *via* a cone mask and the isoflurane level was adjusted depending on the depth of anesthesia. Animal respiratory rate and body temperature were constantly monitored during the data acquisition period (SA Instruments Inc, Stony Brook, NY, USA). The animals were kept warm throughout the experiment at approximately 38°C. Mice were positioned in a custom-made imaging chamber and the lateral tail vein was cannulated for i.v. administration. For PET imaging a microPET Focus220 (Siemens Medical Solutions, Knoxville, USA) was used. [^{11}C]MC113 (28 $\pm$ 10 MBq; containing <0.1 nmol of unlabeled MC113) was injected over approximately 60 s in a volume of 0.1 mL and dynamic emission scans were initiated at start of radiotracer injection. For the experiments in tumor bearing animals [^{18}F]FDG (4 $\pm$ 1 MBq) was injected over approximately 60 s in a volume of 0.1 mL followed by a 20-min static PET scan (see Fig. 2). For all groups of animals, 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 ^{57}Co point source was recorded for 10 min. After completion of the imaging procedure, animals were sacrificed by cervical dislocation while still under deep anesthesia. In tumor bearing animals tumors were

excised and snap frozen in liquid nitrogen and stored at -80°C until Western blot analysis of Pgp expression levels as described in detail elsewhere [20].

2.6. 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 (normalization, decay correction and injection decay correction) was applied to the data. Tumor 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 standardized uptake value ($\text{SUV} = (\text{radioactivity per cubic centimeter/injected radioactivity}) \times \text{body weight}$), were calculated for each VOI and areas under the TACs from 0 to 60 min after radiotracer injection (AUC_{0-60}) were calculated.

2.7. Analysis of metabolites of [^{11}C]MC113

A separate group of FVB wild-type mice ($n=3$), which did not undergo PET scanning, was injected under isoflurane-anesthesia with [^{11}C]MC113 (70 ± 3 MBq in a volume of approximately 0.1 mL) for metabolite analysis. Animals remained under anesthesia, were sacrificed at 30 min after radiotracer injection and a terminal blood sample (0.5 mL) was collected. Blood was centrifuged to obtain plasma ($10000 \times g$, 5 min, 21°C), measured for radioactivity in a gamma counter (Perkin Elmer Instruments, Wellesley, USA) and analyzed for radiolabeled metabolites of [^{11}C]MC113 using a modified version of a previously described solid-phase extraction assay [22]. In brief, arterial plasma was diluted with water (0.5 mL) and acidified with 5M aq. HCl (80 μL) and loaded on a Sep-Pak vac tC18 cartridge (Waters Corporation, Milford, USA), which had been preactivated with methanol (3 mL) and water (5 mL). The cartridge was first washed with water (4 mL) and then eluted with methanol (4 mL). Radioactivity in all three fractions (plasma, water, methanol) was measured in the gamma counter. All three fractions were then further analyzed by thin-layer chromatography (TLC) using Merck silica gel 60 F₂₅₄ plates with ethyl acetate as mobile phase. TLC plates were analyzed using a phosphor imager (Cyclone, Packard Instruments, Meriden, CN, USA). For validation of the solid-phase extraction assay, [^{11}C]MC113 dissolved in water or mouse plasma (0.5 mL) was subjected to the solid-phase extraction procedure showing that all radioactivity was quantitatively recovered in the methanol fraction.

2.8. Rhodamine 123 efflux inhibition assay

Half-maximum inhibitory concentrations (IC_{50}) were determined in a modification of a previously described method [23]. Briefly, cells expressing wild-type Pgp (CCRF-CEM T lymphoblast cell line designated CCRFvcr1000) [24] were sedimented, the supernatant was removed by aspiration, and the cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing rhodamine 123 at a final concentration of 0.2 $\mu\text{g/mL}$ (0.53 μM). Cells were loaded with fluorochrome for 30 min at 37°C . Tubes were chilled on ice and cells were harvested at $500 \times g$ in an Eppendorf 5403 centrifuge (Eppendorf, Hamburg, Germany). The cell pellet was washed with ice cold DMEM medium (pH 7.4) and again centrifuged at $500 \times g$. After resuspension, aliquots of the cell suspension, each containing approximately 1×10^6 cells were transferred to individual fluorescence-activated cell sorter (FACS) tubes and again centrifuged. Supernatants were removed and the pellets were resuspended individually in prewarmed DMEM medium (pH 7.4) containing either no inhibitor or inhibitor (MC113, MC70, MC18, elacridar hydrochloride, tariquidar dimesylate) at various concentrations in DMSO. Each inhibitor concentration was measured in duplicate.

Right after resuspending cells each individual tube was placed in a temperature controlled unit and cell associated fluorescence was monitored over a period of 5 min with a flow cytometer (FACSCalibur, Becton Dickinson, Vienna, Austria). Exponential curves were fitted to the data points and first order rate constants were determined according to the equation:

$$y = a \cdot e^{-(kt)} + c,$$

whereby y is the fluorescence at time t , a is the initial loading, e is the Euler number, k is the first order rate constant, t is the time in seconds and c is the background fluorescence of cells at infinite time. The first order rate constants k , which are independent of initial loading, were used to generate concentration-response curves from which the IC₅₀ values were calculated as 50% occupancy values using the method of least squares using nonlinear regression analysis using the solver add in of the excel software package. At least three individual experiments were performed in duplicate.

2.9. Statistical analysis

Differences between tumor and brain AUC₀₋₆₀ values in scan 1 and scan 2 were analyzed by a two-sided Wilcoxon matched pairs signed rank test using PRISM 5 software (GraphPad Software Inc., La Jolla, CA, USA). Differences in AUC₀₋₆₀ values between FVB wild-type and *Mdr1a/b*^(-/-) mice were analyzed using a Mann Whitney test. The level of statistical significance was set to $P < 0.05$.

3. Results

3.1. Radiosynthesis of [¹¹C]MC113

O-desmethyl-MC113 (MC70, free base), which had been synthesized as described before [19], was reacted with [¹¹C]methyl triflate in DMF/acetone containing aq. NaOH to afford [¹¹C]MC113 in a decay-corrected radiochemical yield of 6±4% ($n=20$), based on [¹¹C]methane, in a total synthesis time of approximately 34 min. Addition of DMF was required as MC70 was poorly soluble in acetone alone. Radiochemical purity of [¹¹C]MC113, formulated for i.v. injection into animals, was >98% and specific activity at end of synthesis was >700 GBq/μmol. Identity of [¹¹C]MC113 was confirmed by HPLC coinjection with unlabeled MC113.

3.2. Brain distribution of [¹¹C]MC113 in wild-type and *Mdr1a/b*^(-/-) mice

Single PET scans with [¹¹C]MC113 were performed in FVB wild-type and *Mdr1a/b*^(-/-) mice ($n=3$ per group) (Fig. 3). TACs of [¹¹C]MC113 were higher in *Mdr1a/b*^(-/-) than in wild-type mice (Fig. 3A). Accordingly, brain AUC₀₋₆₀ values were higher in *Mdr1a/b*^(-/-) than in wild-type mice although statistical significance was not reached (mean±SD AUC₀₋₆₀, *Mdr1a/b*^(-/-): 88±7 min, wild-type: 62±6 min, $P=0.100$, Mann Whitney test) (Fig. 3C). For comparison, for [¹¹C]tarividar mean brain AUC₀₋₆₀ values were 9±1 min in wild-type and 17±2 min in *Mdr1a/b*^(-/-) mice (Fig. 3C). In a separate group of wild-type mice which did not undergo PET scanning ($n=3$), radiolabeled metabolites of [¹¹C]MC113 in plasma were analyzed using a solid-phase extraction assay combined with radio-TLC analysis of individual solid-phase extraction fractions. At 30 min after injection of [¹¹C]MC113, 91±2% of total radioactivity in mouse plasma was in the form of unmetabolized [¹¹C]MC113.

3.3. Evaluation of [^{11}C]MC113 in murine tumor model

Two consecutive PET scans were performed with [^{11}C]MC113, before and after administration of unlabeled tariquidar (15 mg/kg), in mice bearing both low Pgp expressing EMT6 tumors and high Pgp expressing EMT6Ar1.0 tumors. Pgp overexpression in EMT6Ar1.0 relative to EMT6 tumors was confirmed by Western blot analysis of excised tumors collected at the end of PET imaging (Fig. 4). In Figure 2, a diagram of the study set-up is shown. EMT6 and EMT6Ar1.0 tumors were clearly visible in the PET images (Fig. 5C). Both in scan 1 and scan 2, TACs of [^{11}C]MC113 were higher in EMT6 than in EMT6Ar1.0 tumors (Fig. 5A,B). However, AUC_{0-60} values were not significantly different between the two tumor types, both in scan 1 and scan 2 (mean $\pm$ SD AUC_{0-60} , scan 1: EMT6: 76 ± 19 min, EMT6Ar1.0: 65 ± 6 min, $P=0.313$; scan 2: EMT6: 90 ± 23 min, EMT6Ar1.0: 69 ± 8 min, $P=0.063$, Wilcoxon matched pairs signed rank test) (Fig. 5D). In response to tariquidar administration during scan 1, TACs in EMT6Ar1.0 and EMT6 tumors showed modest increases (Fig. 5A). However, for both tumor types AUC_{0-60} values in scan 2 were not significantly different as compared with scan 1 (Fig. 5D). In brain, peak radioactivity uptake (1.7 ± 0.2 SUV at 9 min after radiotracer injection) was comparable with that in tumors. However, brain TACs differed from tumor TACs in that activity cleared from brain, whereas activity continued to rise in tumor tissue during the time course of the PET scan. Comparable to tumor tissue, there was a modest increase in brain TACs in response to tariquidar administration (Fig. 5A). Brain AUC_{0-60} values were higher in scan 2 than in scan 1, although statistical significance was not reached (mean $\pm$ SD AUC_{0-60} , brain: scan 1: 68 ± 7 min, scan 2: 74 ± 7 min, $P=0.063$, Wilcoxon matched pairs signed rank test) (Fig. 5D).

3.4. Inhibition of rhodamine 123 efflux by MC113

The inhibitory effects of MC113 and MC70 on Pgp-mediated efflux transport of rhodamine 123 were measured in CCRFvcr1000 cells and compared with MC18, elacridar and tariquidar (Fig. 6). In Figure 6A, log concentration-response curves are shown for all 5 inhibitors. Figure 6B gives mean ($\pm$ SD) IC_{50} values for all 5 inhibitors from at least 3 independent experiments. The rank order of potency was as follows: elacridar (IC_{50} : 3.2 ± 0.5 nM) > tariquidar (IC_{50} : 8.5 ± 2.5 nM) > MC18 (IC_{50} : 351 ± 40 nM) > MC113 (IC_{50} : 375 ± 60 nM) > MC70 (IC_{50} : 2229 ± 207 nM).

4. Discussion

In search for a PET tracer which binds to Pgp without being transported by it, we labeled MC113 [19], a structural analogue of tariquidar and elacridar (Fig. 1), with ^{11}C and investigated the suitability of [^{11}C]MC113 to visualize Pgp expression levels *in vivo* with PET by using two different approaches. As a first approach we performed PET scans with [^{11}C]MC113 in wild-type mice and in mice in which Pgp was genetically knocked out (*Mdr1a/b*^{-/-}) (Fig. 3). In wild-type mice, brain uptake of activity (AUC_{0-60}) after injection of [^{11}C]MC113 was approximately 7 times higher than for [^{11}C]tariquidar (Fig. 3C), which suggested that active efflux transport played a less important role in limiting brain distribution of [^{11}C]MC113 as compared with [^{11}C]tariquidar. However, in *Mdr1a/b*^{-/-} mice, brain activity uptake of [^{11}C]MC113 was modestly increased as compared with wild-type mice, similar to what has been previously observed for [^{11}C]tariquidar, which could mean that [^{11}C]MC113 was weakly transported by Pgp at the BBB [9]. These findings indicate that, like [^{11}C]tariquidar, [^{11}C]MC113 cannot provide a measurable Pgp binding signal at the murine BBB. If [^{11}C]MC113 had shown Pgp-specific binding, PET signal in *Mdr1a/b*^{-/-} mice, which lack Pgp at the BBB, would have been decreased rather than increased relative to wild-type animals.

As a second approach to characterize [^{11}C]MC113 *in vivo*, we employed a preclinical tumor model in which mice were implanted in one flank with a low Pgp expressing (EMT6) and in the other flank with a high Pgp expressing breast tumor graft (EMT6Ar1.0). This model was chosen as it has previously been successfully used to demonstrate Pgp-specific binding of [^{11}C]tariquidar *in vivo*, which had shown approximately 60% higher AUC₀₋₆₀ values in EMT6Ar1.0 than in EMT6 tumors [20]. The ability of [^{11}C]tariquidar to visualize Pgp expression levels in tumors as opposed to the BBB was explained in a way that Pgp expression levels may have been much higher in tumors than in brain vascular endothelial cells and that Bcrp was not overexpressed in tumors, presumably leading to a lesser extent of Bcrp-mediated efflux transport of [^{11}C]tariquidar [20]. Contrary to the observations made with [^{11}C]tariquidar, tumor uptake of [^{11}C]MC113 was not increased but rather slightly decreased in EMT6Ar1.0 as compared with EMT6 tumors without reaching statistical significance (Fig. 5). Due to the fact that tumors were implanted bilaterally into animals each animal served as its own control, so that activity concentrations in the two tumor types could be directly compared without the need to consider blood activity concentrations. Moreover, analysis of radiolabeled metabolites by a combined solid-phase extraction/radio-TLC assay had revealed that >90% of activity in mouse plasma at 30 min after radiotracer injection was in the form of unmetabolized [^{11}C]MC113, which suggests that PET signal measured in tumor tissue was mainly due to unmetabolized tracer.

To assess Pgp-specificity of PET signal in tumor, we administered a high dose (15 mg/kg) of cold tariquidar during the first PET scan with [^{11}C]MC113 (Fig. 2). In the case of [^{11}C]tariquidar, administration of cold tariquidar during scan 1 had resulted in immediate increases in activity uptake, in EMT6Ar1.0 and EMT6 tumors as well as in brain, implying that [^{11}C]tariquidar was to some extent also actively effluxed by Pgp in the tumor model apart from binding to Pgp [20]. Compared with [^{11}C]tariquidar, administration of cold tariquidar led for [^{11}C]MC113 to only very small increases in tumor and brain activity uptake both in scan 1 and scan 2 (Fig. 5), again supporting the assumption that [^{11}C]MC113 was transported to a lesser extent by Pgp *in vivo* than [^{11}C]tariquidar. However, the lack of increased *in vivo* binding of [^{11}C]MC113 in EMT6Ar1.0 as compared with EMT6 tumors suggests that [^{11}C]MC113 is as opposed to [^{11}C]tariquidar not suitable to visualize Pgp expression in the tumor model. Interestingly, the kinetics of [^{11}C]MC113 were different in tumors as compared with brain in that [^{11}C]MC113 accumulated over time in tumor tissue whereas it was washed out from brain tissue (Fig. 5). As this behavior of [^{11}C]MC113 was observed both in low and high Pgp expressing tumors, before as well as after administration of tariquidar, it seems very unlikely that it was related to a Pgp-specific mechanism.

What could be the reason that [^{11}C]MC113 failed to provide a Pgp-specific binding signal *in vivo*? We compared the potencies of MC113 and MC70 to inhibit transport of the fluorescent Pgp substrate rhodamine 123 in Pgp expressing cells with those of MC18, elacridar and tariquidar (Fig. 6). Contrary to results obtained previously in the Caco-2 transport inhibition assay using [^3H]vinblastine as substrate in which MC70 was shown to be more potent than elacridar [19], our data showed that MC113, MC70 and MC18 were up to two orders of magnitude less potent than tariquidar and elacridar in inhibiting rhodamine 123 transport (Fig. 6B). The exact reasons why the Caco-2 transport inhibition and rhodamine 123 efflux inhibition assays gave different rank orders of inhibitory potencies for our compounds are not known, but could be related to the fact that different substrates were used in the two assays (vinblastine *versus* rhodamine 123) and that Caco-2 cells are known to express, in addition to Pgp, a range of other efflux transporters (e.g. BCRP, MRP2) [25]. When assuming that the ability to inhibit substrate transport reflects the Pgp binding affinity of an inhibitor, our rhodamine 123 efflux inhibition data could mean that the Pgp binding affinity of MC113 might be too low in relation to the density (B_{max}) of Pgp at the murine BBB to achieve a Pgp binding signal with [^{11}C]MC113. Using a quantitative proteomics

approach Kamiie and colleagues reported a $B_{\max}$ of the Mdr1a peptide of 15 fmol/ μ g protein in mouse brain capillaries [26], which translates to a value of 1.5 nM when assuming that the BBB constitutes only about 0.1% of total brain weight and that the protein content of brain capillaries is approximately 10%. This would mean that compounds with subnanomolar Pgp binding affinities would be needed to visualize Pgp at the BBB with PET and to achieve $B_{\max}/K_d$ ratios >1 . Given that unlike previously developed radiolabeled Pgp inhibitors [^{11}C]MC113 did not appear to be transported to a high extent by Pgp at the BBB *in vivo* and also showed high metabolic stability *in vivo*, the compound might serve as a lead for future development of Pgp inhibitor PET tracers with improved Pgp binding affinities.

5. Conclusion

We evaluated the radiolabeled Pgp inhibitor [^{11}C]MC113 with small-animal PET imaging in wild-type and *Mdr1a/b*^(-/-) mice as well as in a low and high Pgp expressing mouse tumor model with respect to its ability to visualize Pgp expression levels. As compared with [^{11}C]tariquidar, [^{11}C]MC113 appeared to be transported *in vivo* to a considerably lesser extent by Pgp and/or Bcrp at the BBB, leading to higher brain uptake of [^{11}C]MC113. However, [^{11}C]MC113 failed to provide a Pgp-specific binding signal both at the mouse BBB and in the tumor model, presumably due to insufficiently high Pgp binding affinity of MC113 in relation to the $B_{\max}$ of Pgp. Therefore our data suggest that [^{11}C]MC113 is not suited as a PET tracer to visualize Pgp expression levels.

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Fig. 1. Chemical structures of Pgp inhibitors discussed in this work. When applicable, ^{11}C -labeling positions are indicated by an asterisk *.

Fig. 2.

Diagram of study set-up used for evaluation of [¹¹C]MC113 in the murine tumor model. Scan 1 (150 min), during which cold 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 cold tariquidar administration. At the end of scan 2, a 20-min static [¹⁸F]FDG PET scan was performed to facilitate definition of tumor ROIs.

Fig. 3. Mean (+SD) brain time-activity curves (A), sagittal PET summation images (0-60 min) (B) and individual brain AUC₀₋₆₀ values (C) of $[^{11}\text{C}]\text{MC113}$ in FVB wild-type and $Mdr1a/b^{-/-}$ mice ($n=3$ per group). PET scans were acquired under isoflurane anesthesia (2.5%), image acquisition time for the shown PET images was 60 min and the injected activities were 24.4 MBq and 12.9 MBq for the shown wild-type and $Mdr1a/b^{-/-}$ mouse, respectively. PET images are set to an intensity scale of 0-3 SUV. The localization of the brain (br) is indicated by a white arrow. For comparison, AUC₀₋₆₀ values of $[^{11}\text{C}]\text{Tariquidar}$ in FVB wild-type and $Mdr1a/b^{-/-}$ mice are also shown in C [9]. Horizontal lines in C represent geometric means $\pm$ SD.

Fig. 4.

Representative Western blot analysis of Pgp (170 kDa) in EMT6 and EMT6Ar1.0 derived tumors excised at the end of PET imaging. Expression of Pgp was assessed using the monoclonal antibody C219. Identical amounts of tumor protein (50 μ g) were loaded in each well. Expression of β -actin was also measured to confirm consistent gel loading. EMT6Ar1.0 whole cell lysate (0.5 μ g protein) with confirmed overexpression of Pgp [20] was used as positive control for Pgp.

Fig. 5. Mean time-activity curves of $[^{11}\text{C}]\text{MC113}$ (SUV+SD, $n=5$) in EMT6 tumors (blue squares), EMT6Ar1.0 tumors (red squares) and brain (black open circles) for scan 1 (A) and scan 2 (B). Tariquidar (15 mg/kg) was administered as an i.v. bolus at 60 min after start of scan 1 (arrow). (C) Representative, transversal PET summation images (30-60 min) of $[^{11}\text{C}]\text{MC113}$ for scan 1 and scan 2. Corresponding $[^{18}\text{F}]\text{FDG}$ images for tumor definition from the same animal are shown in the right column. PET scans were acquired under isoflurane anesthesia (2.5%). Image acquisition times for the PET images shown in C were 30 min ($[^{11}\text{C}]\text{MC113}$) and 20 min ($[^{18}\text{F}]\text{FDG}$) and injected activities were 28.0 MBq and 34.2 MBq for $[^{11}\text{C}]\text{MC113}$ scan 1 and scan 2, respectively, and 3.05 MBq for $[^{18}\text{F}]\text{FDG}$. Localization of EMT6 and EMT6Ar1.0 tumors is indicated by arrows. Radiation scale is expressed as SUV. (D) AUC₀₋₆₀ values in individual EMT6 and EMT6Ar1.0 tumors and whole brain measured from 0-60 min after injection of $[^{11}\text{C}]\text{MC113}$ before (scan 1) and after (scan 2) unlabeled tariquidar administration. Horizontal lines represent geometric means $\pm$ SD.

Fig. 6.

(A) Log concentration-response curves for inhibition of Pgp-mediated efflux transport of rhodamine 123 from CCRFvcr1000 cells for MC113, MC70, MC18, elacridar and tariquidar. For each inhibitor at least 3 independent experiments were performed each in duplicate. Hyperbolic dose-response curves were simultaneously fitted to all data points by the method of least squares. Symbols represent the average of at least 6 individual data points. (B) Mean (+ SD) half-maximum inhibitory concentrations (IC₅₀, nM) of MC113, MC70, MC18, elacridar and tariquidar in the rhodamine 123 efflux inhibition assay from at least 3 independent experiments performed each in duplicate. Note that the y-axis is in logarithmic scale.