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RESEARCH ARTICLE

In Vitro and *In Vivo* Structure–Property Relationship of ^{68}Ga-Labeled Schiff Base Derivatives for Functional Myocardial PET Imaging

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Abstract

Purpose: SPECT (*e.g.*, with ^{99m}Tc-sestamibi) is routinely used for imaging myocardial damage, even though PET could offer a higher spatial resolution. Using the generator-gained isotope ^{68}Ga would allow a rapid supply of the tracer in the diagnostic unit. For this reason, the aim of the study was to develop ^{68}Ga-labeled PET tracers based on different Schiff base amines and to evaluate the cardiomyocyte uptake *in vitro* as well as the biodistribution of the tracers *in vivo*.

Procedures: Fifteen different Schiff bases (basing on 3 different backbones) were synthesized and labeled with ^{68}Ga. Lipophilicity varied between 0.87±0.24 and 2.72±0.14 (logD value). All tracers were positively charged and stable in plasma and apo-transferrin solution. *In vitro* uptake into cardiomyocytes was assessed in HL-1 cells in the absence and presence of the ionophor valinomycin. *In vivo* accumulation in the heart and in various organs was assessed by small animal PET imaging as well as by *ex vivo* biodistribution. The results were compared with ^{99m}Tc-sestamibi and ^{18}F-flurpiridaz.

Results: All cationic Schiff bases were taken up into cardiomyocytes but the amount varied by a factor of 10. When destroying the membrane potential, the cellular uptake was markedly reduced in most of the tracers, indicating the applicability of these tracers for identifying ischemic myocardium. PET imaging revealed that the *in vivo* myocardial uptake reached a constant value approximately 10 min after injection but the intracardial amount of the tracer varied profoundly (SUV 0.46 to 3.35). The most suitable tracers showed a myocardial uptake which was comparable to that of ^{99m}Tc-sestamibi.

Conclusions: ^{68}Ga-based Schiff bases appear suitable for myocardial PET images with uptake comparable to ^{99m}Tc-sestamibi but offering higher spatial resolution. By systematical variation of the backbone and the side chains, tracers with optimal properties can be identified for further clinical evaluation.

Electronic supplementary material The online version of this article (doi:10.1007/s11307-014-0750-3) contains supplementary material, which is available to authorized users.

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Key words: Ga-labeling, Schiff bases, Myocardial imaging, PET, Lipophilicity
Abbreviations: HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; PBS, Phosphate-buffered saline; SUV, Standardized uptake value

Introduction

Coronary artery disease is the most common cause of death in industrial nations [1]. Over longer periods, arteriosclerotic plaques in the coronary arteries can reduce the myocardial supply of O_2 and nutrients. Early noninvasive detection of myocardial damage by insufficient O_2 supply to the tissue would be helpful and (even in the case of infarction) detailed noninvasive imaging could facilitate adequate treatment.

Imaging of coronary artery disease is routinely performed either by the minimal-invasive coronary catheterization with contrast agent or by noninvasive myocardial scintigraphy using single-photon emission computed tomography (SPECT) with ^{99m}Tc-radiopharmaceutials such as ^{99m}Tc-sestamibi or ^{99m}Tc-tetrofosmin. ^{99m}Tc ($t_{1/2}$=6 h) is produced in a generator and therefore continuously available at the clinical laboratory. The tracer is accumulated in the intact cardiomyocyte due to its positive charge. Another myocardial SPECT tracer is ^{201}Tl ($t_{1/2}$=73 h) that acts as an analog of K^+ which is taken up into intact cardiomyocytes. The major disadvantage of this isotope (besides its rather long half-life) is that it is produced in a cyclotron which reduces its availability. Compared to SPECT, the use of positron emission tomography (PET) might have several advantages, especially the higher spatial resolution and the higher sensitivity [2]. Several attempts have been undertaken to use PET for myocardial diagnostics which allows a more detailed regional analysis. As a disadvantage, some of the positron emitters suitable for cardiac PET (^{13}N, ^{18}F, ^{15}O) [3] need a cost- and logistic-challenging cyclotron. ^{13}N-ammonia ($t_{1/2}$=10 min) is preferentially a perfusion marker. However, it can be taken up into the myocytes forming ^{13}N-NH_4^+. In the cardiomyocytes, it can be metabolized into glutamine leading to an accumulation in vital cells (however, due to its relatively short half-life, the signal from glutamine is rather weak). ^{15}O-H_2O ($t_{1/2}$=2 min) distributes freely in the intra- and extracellular space but does not accumulate in vital cells specifically. Therefore, ^{15}O-H_2O is a pure perfusion marker. ^{18}F-flurpiridaz (^{18}F-BMS-747158-02, $t_{1/2}$=110 min) is a structural analog of pyridaben which has a very high affinity to the mitochondrial complex I in cardiomyocytes [4]. For this reason, it accumulates in vital cells very effectively. The major disadvantage of this compound is the isotope production in a cyclotron. Positron emitters derived from radionuclide generators may be favorable. One possibility would be the use of ^{82}Sr/^{82}Rb generator system. ^{82}Rb ($t_{1/2}$=75 s) can be used as a myocardial perfusion marker, but due to its short half-life, practically no specific accumulation in vital cells takes place. Alternatively, a ^{68}Ge/^{68}Ga radionuclide generator can be used which constitutes of a long-living parent (^{68}Ge, $t_{1/2}$= 271 days), which decays to the short-living daughter (^{68}Ga, $t_{1/2}$=67.7 min), which again decays to stable ^{68}Zn [5]. Radiopharmaceutical chemistry of ^{68}Ga has been analyzed extensively during the last years [6, 7], and several PET imaging applications (*e.g.*, for neuroendocrine tumors) have been described (for recent reviews, see [6, 8]). By using this generator system instantaneously, kit-type tracers would be available in the clinical setting (comparable to ^{99m}Tc).

Consequently, ^{68}Ga could be a suitable isotope to be used in PET tracers for imaging myocardial cell damage. First attempts have been undertaken by Green *et al.* [9] who developed different lipophilic radiopharmaceuticals for myocardial imaging. First, neutral tripodal Ga^{3+}-tris(salicylaldimines)-gallium complexes which form a pseudo-octahedral $N_3O_3^{3-}$-coordination sphere around a Ga^{3+} center have been investigated [9, 10]. Those tracers showed high lipophilicity (as indicated by high logD values ranging from 2.5 to 3.1), and high initial myocardial uptake, but suffered from a rapid myocardial clearance due to fast blood-clearance as well as a strong liver uptake due to their lipophilicity. To overcome these problems, tetra-amino Schiff bases which form monocationic radiocomplexes with ^{68}Ga have been synthesized. The complexes are mostly lipophilic and positively charged and reveal high uptake and retention in the intact myocardium [11–14]. *In vivo*, some tracers showed to be promising for myocardial imaging having a heart uptake of about 2 % of the injected dose at 120 min p.i. and high heart-to-liver ratio in rats [11]. Those compounds are supposed to be retained intracellularly due to their positive charge, which is essential to obtain marked heart-to-blood, heart-to-lung, and heart-to-liver ratios. Despite these promising results, no relationship between lipophilicity or targeting group and myocardial uptake in these exemplarily described tracers could be identified.

For this reason, in the present study, a broad spectrum of Schiff-base complexes was synthesized and ^{68}Ga-labeled. By introducing alternative substitution pattern of the used salicylaldehydes, the physicochemical properties (*e.g.*, lipophilicity) of the molecules can be systematically modified. All derivatives analyzed were base on one of three Schiff-base backbones carrying salicylaldehydes with different substitutes. In order to assess the role of the backbone, the same aldehydes were coupled to all three backbones and the

impact on physicochemical properties as well as on cellular accumulation was analyzed. On the other hand, different salicylaldehydes containing bromine-, alkoxy-, nitro-, alkyl-, and *N*-ethyl-groups were coupled to the same backbone in order to assess the impact of the substitutes for the molecular properties and cell uptake.

The aim of the study was to analyze the structure–property relationship of systematically modified derivatives and to evaluate the cardiomyocyte uptake *in vitro* as well as the biodistribution of the tracers *in vivo*. The results were compared with the clinically used tracers ^{99m}Tc-sestamibi and ^{18}F-flurpiridaz.

Materials and Methods

Chemistry

All derivatives were analyzed base on one of three Schiff-base backbones, namely 1,2-bis(3-aminopropylamino)ethane (BAPEN), bis(2,2-dimethyl-3-aminopropyl)ethylenediamine (BAPDMEN), and bis(*N*,*N*′-amino-2,2-dimethylpropane)ethylenediamine (BADED) (Fig. 1a). BAPEN and BADED contain (besides the two terminal primary amines) two secondary amines, whereas BAPDMEN contains tertiary amines showing lower chemical reactivity. These tetra-amino backbones were condensed with salicylaldehydes to form the Schiff base amines (Fig. 1b). The salicylaldehydes varied in their substitution pattern. Since the tracer should enter the cells by nonionic diffusion, aldehydes carrying lipophilic groups (bromine-, methyl-groups) were inserted. On the other hand, polar groups could increase the retention within the cell and hence improve cellular accumulation. Therefore, aldehydes with *N*-ethyl-groups were synthetized. Finally, the tracer which enters the myocardium should possibly not accumulate in the liver in order to give a high heart-to-liver contrast. Since previous studies showed that ether groups reduce liver uptake, aldehydes with methoxy- or ethoxy-groups were synthetized.

In order to test the impact of different substitutes (Table 1) on the physicochemical properties and the uptake/retention in cardiomyocytes, these different salicylaldehydes were coupled to the same backbone BADED (BADED-1 to BADED-9; Fig. 2). In the second series of experiments, the role of the backbone was addressed. Therefore, the same salicylaldehydes derivatives (1) two methoxy-groups, pos. 4 and 6, (2) one methoxy-group, pos. 3, and (3) bromine, pos. 5, were tested with all three backbones (Fig. 2), providing information on the role of backbone structure. The systematic variation of the structure includes also those derivatives described previously by others [11–16].

Synthesis of the backbones and the Schiff base amines was performed according to literature [11, 12, 17]. Details of the synthesis and the characterization data are available in the Supplementary Material.

Radiosynthesis

^{68}Ga was eluted from the ^{68}Ge/^{68}Ga-generator with 6 ml 0.1 N HCl and trapped on an acidic cation exchanger. Metal impurities (*e.g.*, Zn, Fe, Ti, and Ge) were removed by usage of 1 ml washing solution (N1, 80 % acetone + 0.15 M HCl) [5]. Subsequent elution from the cation exchange resin was performed *via* two different ways.

Labeling in Organic Media Purified ^{68}Ga was desorbed from the cation exchange resin with 600 µl of 98 % acetone and 2 % acetylacetone as described before [18]. The obtained solution was evaporated to dryness resulting in an orange-colored pellet which was dissolved in 400 µl $CHCl_3$. Twenty microliters of the Schiff base amines dissolved in ethanol (1 mg/ml) was added and heated

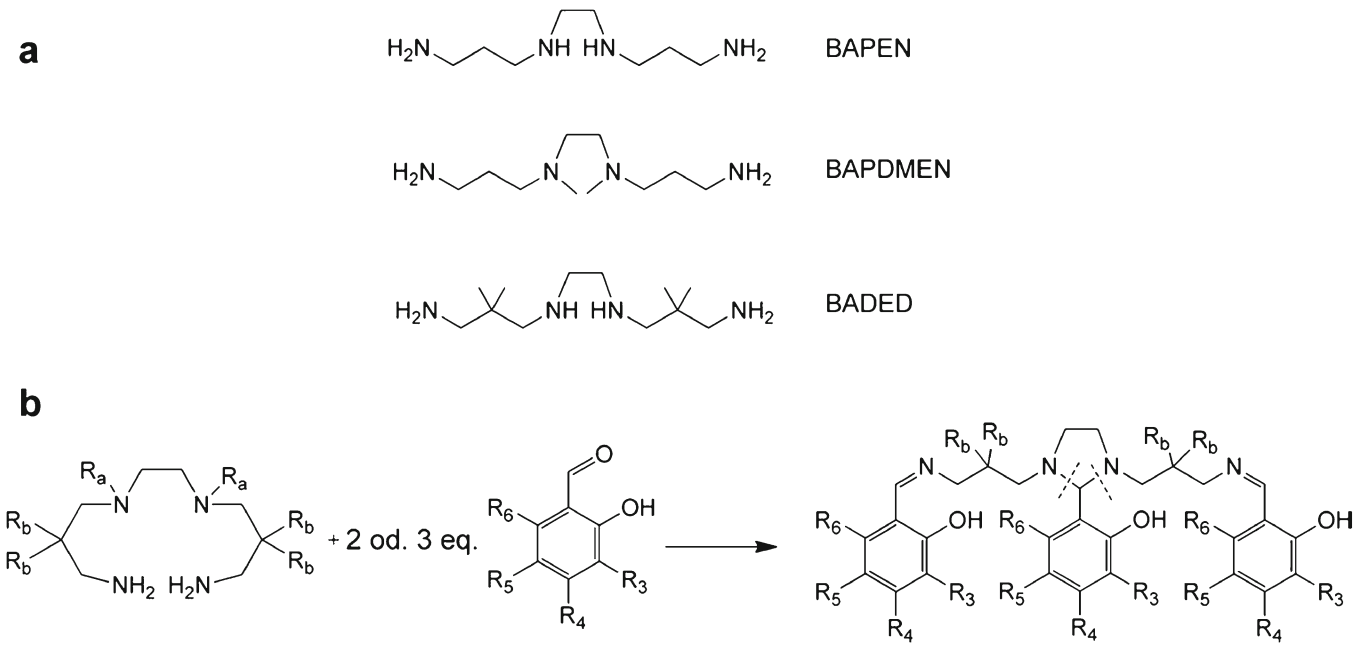

Fig. 1. **a** Molecular backbones of the Schiff bases used. **b** General structural formula for the Schiff base amines. Substituents are specified in Table 1.

Table 1. Substituents R of the general backbone structures (Fig. 1a) of the Schiff base amine and partition coefficients between octanol and PBS (logD) of these derivatives

Compound	Backbone	R3	R4	R5	R6	logD
^{99m}Tc-sestamibi						0.64±0.25
^{68}Ga-BAPEN-3	BAPEN	H	H	Br	H	0.87±0.24
^{68}Ga-BAPDMEN-1	BAPDMEN	H	OCH_3	H	OCH_3	1.07±0.27
^{68}Ga-BADED-2	BADED	OCH_3	H	H	H	1.16±0.09
^{68}Ga-BAPDMEN-2	BAPDMEN	OCH_3	H	H	H	1.16±0.05
^{68}Ga-BADED-6	BADED	H	H	NO_2	H	1.30±0.16
^{68}Ga-BADED-7	BADED	H	$N(CH_2CH_3)_2$	H	H	1.46±0.06
^{68}Ga-BAPDMEN-3	BAPDMEN	H	H	Br	H	1.48±0.14
^{68}Ga-BADED-4	BADED	H	H	H	H	1.60±0.03
^{68}Ga-BAPEN-1	BAPEN	H	OCH_3	H	OCH_3	1.60±0.15
^{68}Ga-BAPEN-2	BAPEN	OCH_3	H	H	H	1.71±0.17
^{18}F-flurpiridaz						1.76±0.31
^{68}Ga-BADED-1	BADED	H	OCH_3	H	OCH_3	1.92±0.14
^{68}Ga-BADED-5	BADED	OCH_2CH_3	H	H	H	2.03±0.09
^{68}Ga-BADED-9	BADED	H	H	$C(CH_3)_3$	H	2.43±0.12
^{68}Ga-BADED-3	BADED	H	H	Br	H	2.49±0.07
^{68}Ga-BADED-8	BADED	OCH_3	H	Br	H	2.72±0.14

For comparison, the value of ^{99m}Tc-sestamibi is shown. The tracers are arranged in the order of their lipophilicity (logD)

to 80 °C for 10 min to ensure complete complex formation. The ^{68}Ga-complexes were obtained as solids which were dissolved in isotonic NaCl containing 10 % ethanol.

Labeling in Aqueous Media Four hundred microliters of an acetone-HCl mixture (97.6 % acetone/0.005 M HCl) was used to elute the cation exchange column [5, 19]. This eluate was added to 400 µl 0.1 N HEPES buffer and 20 µl of the corresponding Schiff base amine in ethanol (1 mg/ml). The reaction mixture was heated at 80 °C for 10 min.

Radiochemical purity of ^{68}Ga-Schiff base complexes was proven by thin-layer chromatography (SG 60 F254 plates, Merck, Darmstadt, Germany; analysis: Instant Imager Canberra Packard, Schwadorf, Austria). A mixture of 90 % MeOH and 10 % isotonic NaCl solution was used as mobile phase. Unrcomplexed $^{68}Ga^{3+}$ or $^{68}Ga(acac)_3$ remained at the starting line (R_f=0), whereas the reaction product was found at R_f=0.9. In order to verify the identity of the ^{68}Ga-Schiff base complexes, comparative radioHPLC and HPLC analyses were performed. Details of the method and results are available in the Supplementary Material.

Purification of the ^{68}Ga-complexes was performed *via* solid-phase extraction. Therefore, a Waters C-18 light cartridge (Sep-Pak C18 Plus Light Cartridge, 130 mg, Waters Corp., Milford MA, USA) was preconditioned with 1 ml ethanol followed by 3 ml water. The ^{68}Ga-Schiff base complex was passed through the resin, trapped on the cartridge, and washed with another 1 ml water to get rid of unreacted Ga^{3+}. The ^{68}Ga-Schiff base complex could then be eluted with about 1 ml ethanol. Ethanol was removed by heating the open vial for about 15 min at 85 °C. The efficiency of the radiotracer purification using C18 cartridges has been analyzed by HPLC. Details of the method and results are available in the Supplementary Material.

Lipophilicity

Determination of the lipophilicity was performed as described before [17]. In brief, purified ^{68}Ga-Schiff base complexes were dissolved in 700 µl PBS buffer and mixed with 700 µl octanol. The mixture was shaken (shake-flask method). After centrifugation, the two phases were separated, and aliquots were taken and spotted on a piece of paper. Their radioactivity was measured on an Instant Imager (Canberra Packard). Afterwards, 400 µl of the octanol phase were transferred into a vial, diluted with 300 µl fresh octanol, and mixed with 700 µl PBS buffer. The mixture was centrifuged again, and this procedure was performed in total three times. The first extraction was excluded from analysis in order to minimize contamination of the sample with uncomplexed ^{68}Ga or salts. The partition coefficient was calculated as the ratio of the radioactivity of octanol to water phase in the second and third back extraction.

Stability Studies

In vitro stability was performed following as previously described [17]. Purified ^{68}Ga-Schiff bases (~5 MBq) were incubated at 37 °C in 400 µl isotonic NaCl, 400 µl of apo-transferrin solution (1 mg/ml PBS-buffer), and 400 µl human serum (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) using a thermoshaker. Samples were taken after 1, 10, 20, 30, 50, and 60 min. Radiochemical purity was analyzed by radio-thin layer chromatography (SG 60 F254 and RP-18 F254s plates, Merck, Darmstadt, Germany; Instant Imager, Canberra Packard). All experiments were run in duplicate for each compound.

Charge

Paper electrophoreses was performed with a Pharmacia LKB Biotechnologie Multiphore II electrophorese chamber developed with 0.4 N tris(hydroxymethyl)aminomethane (TRIS)-buffer at pH 7.0 and 50 vol.% ethanol (to ensure solubility). A constant voltage of 600 V was applied (5.4 mA and 3 W) for 2 h. Radioactivity distribution on the stripe was determined using an Instant Imager (Canberra Packard).

Cell Uptake In Vitro

Mouse HL-1 myocardium cells were used in all experiments [20]. Cells were grown in Claycomb medium supplemented with 10 %

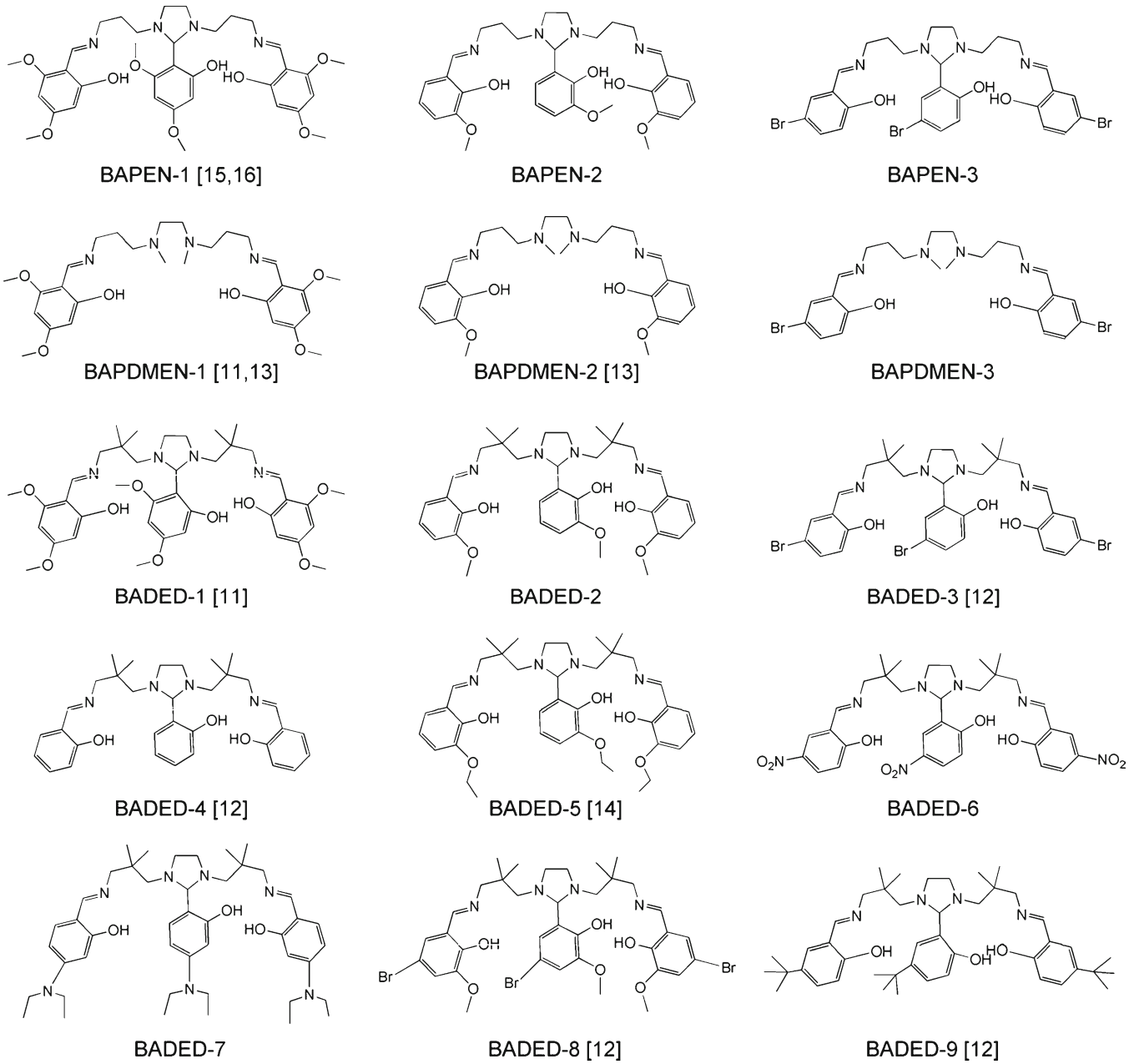

Fig. 2. Synthesized Schiff base amines. Previously described complexes are marked with their reference.

fetal calf serum (FCS) at 37 °C under 5 % CO_2 atmosphere and subcultivated once per week. Prior to the experiments, cells were transferred to test tubes and dispersed in medium. At least 1 million cells were used per tube. In order to assess membrane potential dependency of the uptake of the ^{68}Ga-Schiff base complexes into the myocardial cells, the uptake was determined in the presence and absence of the K^+ ionophor valinomycin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Valinomycin destroys the mitochondrial membrane potential leading to a breakdown of ATP formation (comparable to the situation of myocardial ischemia). The lack of ATP will lead to a loss of the negative cellular membrane potential which will in return reduce the accumulation of a positively charged tracer. Valinomycin was added to every second tube (final concentration 1 μM) prior to tracer addition. The tracers were used at an activity of ~5 MBq ^{68}Ga-Schiff base complex.

The cells were incubated with the tracer (with or without valinomycin) in medium for 30 min at 37 °C. After incubation, cell suspension was separated by centrifugation (without washing) and the radioactivity of cells and supernatant were measured in a dose calibrator (M2316, Messelektronik Dresden GmbH, Germany).

Animal Experiments

All experiments had previously been approved by the regional animal ethics committee and were conducted in accordance with the German Law for Animal Protection.

μPET and Data Reconstruction Animal imaging experiments were performed in Sprague Dawley rats (body weights 235–560 g, male and female) under isoflurane anesthesia (2 % isoflurane, 98 % O_2). For injection, the purified ^{68}Ga-Schiff base complexes were dissolved in approximately 0.7 ml isotonic saline containing 10 % ethanol to ensure solubility. The radiotracer was administered as a bolus injection (dose 17.8±5.7 MBq) *via* the tail vein. PET imaging was performed on a μPET Focus 120 small animal PET imager (Siemens/Concorde, Knoxville, USA). During PET measurements, the animals laid supine and the scanning position was placed over the chest. Following a 15-min transmission scan with an external ^{57}Co source, dynamic PET images were acquired in 2D mode. The PET listmode data were histogrammed into 19 frames and reconstructed using OSEM2D algorithm. Time activity curves (TAC) were obtained with varying time frames (20 s–5 min) for a total measuring interval of 60 min. Three-dimensional regions of interest were manually drawn around the myocardium to define the volume of interest (VOI) and standardized uptake values (SUV) were calculated. For comparison, ^{18}F-flurpiridaz was injected at a dose of 18.5 MBq.

Biodistribution Study Ex Vivo Since ^{68}Ga-BADED-2 and ^{68}Ga-BAPDMEN-2 showed high myocardial and low liver uptake in the PET studies, these tracers were selected for biodistribution studies. Injected activities were 9.71±2.42 MBq for the ^{68}Ga-Schiff base complexes, 8.01±0.32 MBq ^{18}F-flurpiridaz, and 24.61±2.06 MBq for ^{99m}Tc-sestamibi. The tracers were injected into the tail vein of Sprague Dawley rats (n=4 per radiotracer, 140–500 g, maleStatistical AnalysisResults are and female). Sixty minutes after injection, animals were sacrificed and organs of interest (lung, blood, liver, spleen, kidney, skeletal muscle, heart, and brain) were surgically removed. Tissue radioactivity was measured by a Gamma-Counter (Wizard®, PerkinElmer). The biodistribution of the labeled compounds was calculated as a percentage of injected dose per gram of tissue (%ID/g).

Statistical Analysis Results are expressed as mean ± SD. Differences between groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at α=5 % for all comparisons. Correlation analysis was performed by calculating the Pearson correlation coefficient.

Results

Chemistry

All Schiff base amines were synthesized and characterized by ^{1}H-NMR and electrospray ionization-mass spectrometry. All analysis data are shown in the Supplementary Material.

Radiosynthesis

^{68}Ga labeling of all Schiff base amines led to >87 % labeling yield (t=10 min, T=80 °C; see Supplementary Material) in organic and aqueous media. Purification of the ^{68}Ga-Schiff bases was achieved by solid-phase extraction (Waters C-18 light cartridge) resulting in >95 % radiochemical purity. Separation of the byproduct, ligand, and gallium complex by the C18 cartridge was reasonable (see Supplementary Material).

Lipophilicity

Partition coefficients between octanol and PBS (logD) of the ^{68}Ga-Schiff base complexes ranged from 0.87±0.2 to 2.72±0.14 (Table 1).

Stability Studies In Vitro

Figure 3 shows the stability of the ^{68}Ga-Schiff base complexes after 60 min in isotonic saline, apo-transferrin solution, and human serum. Almost all ^{68}Ga-schiff base complexes remain stable in all media (stability >92 %). Only ^{68}Ga-BAPEN-3 and ^{68}Ga-BAPEN-2 showed markedly lower values. Especially for ^{68}Ga-BAPEN-3, the stability in apo-transferrin solution decreased constantly over time down to only 39±2 % (Supplementary Fig. 1).

Electric Net Charge

Analogue to ^{99m}Tc-sestamibi, it is assumed that a cationic charge of the ^{68}Ga-Schiff base complexes favors the retention in the myocardial cells due to the negative mitochondrial potential. Paper electrophoreses proofed that all the ^{68}Ga-Schiff base complexes migrated to the cathode at pH 7.0 (examples Supplementary Fig. 2) and therefore have a positive charge at physiological pH.

Cell Uptake In Vitro

A suitable tracer for assessing the vitality of cardiomyocytes should fulfill two criteria as follows: (1) it should enter and

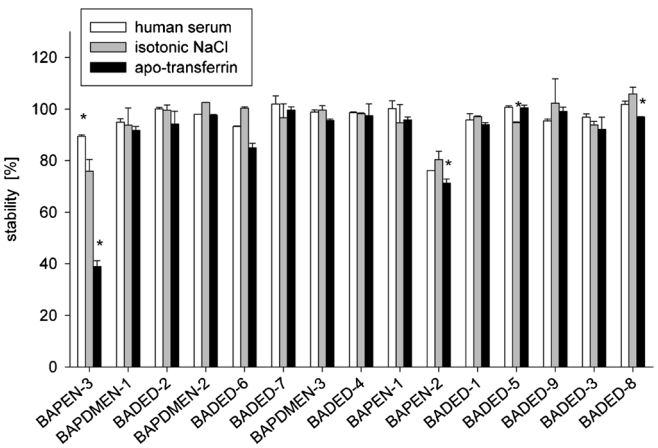

Fig. 3. Stability of ^{68}Ga-Schiff bases in human serum, isotonic saline, and apo-transferrin solution after 60 min. Tracers are arranged in the order of their lipophilicity (logD value). (*) $p<0.05$.

accumulate in the vital myocytes, and (2) in case of cell damage, accumulation should be markedly reduced. In order to measure the passive uptake of the compounds, cells were incubated with the tracer in the presence or absence of valinomycin and the distribution between cells and supernatant was determined. The ratio of the cellular uptake with and without valinomycin can be considered as a measure reflecting cellular damage. Figure 4a clearly reveals that the tracers were accumulated pronouncedly different, varying from 3 % to almost 47 % of the tracer found in the cell pellet. Treating the cells with valinomycin reduced the cellular uptake of all tracers by 6 to 28 % (Fig. 4b), except ^{68}Ga-BAPEN-3, ^{68}Ga-BAPDMEN-3, and ^{68}Ga-BAPEN-2, which showed no impact of the membrane potential (for some tracers, *e.g.*, ^{68}Ga-BADED-7, the level of significance was not reached due to the limited number of experiments; $n=2$). ^{68}Ga-BADED-1 exhibited the most pronounced decrease in tracer accumulation by 28 % (Fig. 4b), whereas most of the tracers (including ^{99m}Tc-sestamibi as a reference) showed a reduction of about 15 %. The cellular uptake pattern in untreated cells was only weakly correlated with the lipophilicity of the individual tracer (Fig. 4a). ^{68}Ga-Schiff base complexes which had more lipophilic substituents (*e.g.*, bromine in m-position like ^{68}Ga-BADED-3) showed a higher uptake (10.3±2.4 %) than those without a lipophilic substituent (*e.g.*, ^{68}Ga-BADED-4, 2.9±0.7 %). In contrast to this, the addition of the less lipophilic nitro group did not reduce the uptake (^{68}Ga-BADED-6, 7.0±0.4 %). The comparison of the ethyl ether versus the methyl ether substituent indicate that the shorter ether group lead to a higher uptake (^{68}Ga-BADED-5, 3.7±0.7 % *vs.* ^{68}Ga-BADED-2, 8.9±0.8 %). Combining the addition of a lipophilic group with a methyl ether functionality lead to an even higher cellular uptake (^{68}Ga-BADED-8, 11.1±0.3 %). ^{68}Ga-BADED-7 (with the *N*-diethyl, substituent on 4-position) and ^{68}Ga-BADED-9 (*tert*-butylgroup on 5-position) showed markedly high cellular uptake of 46.1±2.1 and 41.4±2.8 %, respectively. The inhibition of cell accumulation by valinomycin showed no systematic dependency on the molecular structure or the lipophilicity (Fig. 4b).

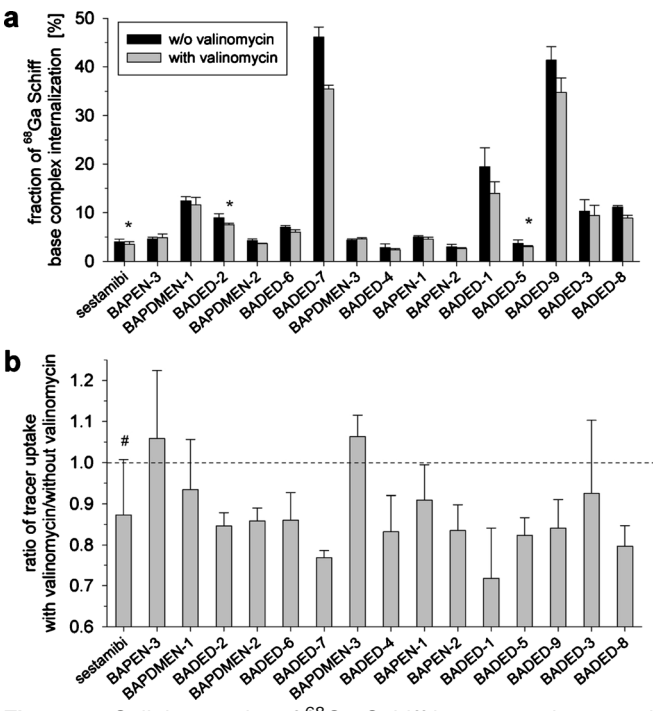

Fig. 4. **a** Cellular uptake of ^{68}Ga-Schiff base complexes and ^{99m}Tc-sestamibi into HL-1 cells (measured by the fraction of tracer internalization) in the absence and presence of valinomycin. **b** Ratio of the cellular tracer concentration in the presence of valinomycin to the tracer concentration in control cells. Tracers are arranged in the order of their lipophilicity (logD value). $n=2$–10; (*) $p<0.05$ with versus without valinomycin; (#) $p<0.05$ different from ratio = 1.0.

In Vivo Experiments

μPET Studies All tracers showed an accumulation in the myocardium during 60 min of PET imaging (Fig. 5a, b). Kinetic analyses indicate that after an initial redistribution phase during the first minutes, a constant myocardial uptake was reached approximately 10 min after injection. This plateau remained almost stable for the whole time of acquisition (60 min) for almost all tracers (Supplementary Fig. 3).

Figure 5c shows the myocardial uptake values of the tracers. Increased uptake in the heart was found for those ^{68}Ga-Schiff base complexes that were substituted in 3-position with a methoxy-group (^{68}Ga-BADED-2, $SUV_{myocardium}=3.4\pm0.5$, ^{68}Ga-BADPDMEN-2, $SUV_{myocardium}=3.0\pm1.1$, and ^{68}Ga-BADED-8, $SUV_{myocardium}=2.7\pm0.9$). In contrast, ethoxy substitution on position 3 leads to a decreased uptake (^{68}Ga-BADED-5, $SUV_{myocardium}=2.3\pm0.7$). Decreased uptake was also observed in ^{68}Ga-Schiff base complexes which were

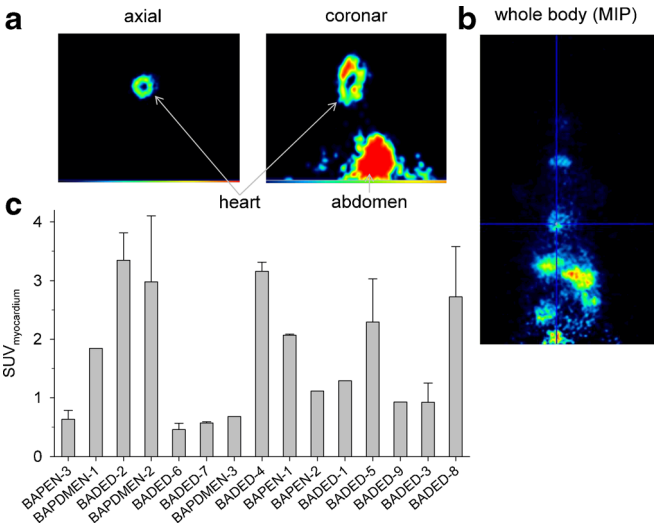

Fig. 5. **a** Example of PET images of the accumulation of ^{68}Ga-BADED-2 in the myocardium and **b** whole body maximum intensity projection (*MIP*) during the interval $t=$ 15–60 min after injection. **c** Standardized uptake values (*SUV*) of the myocardium *in vivo* of the ^{68}Ga-Schiff base complexes after 60 min. Tracers are arranged in the order of their lipophilicity (logD value).

substituted in 5-position, either with electron withdrawing substituents (^{68}Ga-BADED-6, $SUV_{myocardium}$=0.5±0.1) or electron donating substituents (^{68}Ga-BADED-9, $SUV_{myocardium}$=0.93). Also ^{68}Ga-BADED-3, which was derivatized at the 4-position with a bromine, showed less uptake in the myocardium ($SUV_{myocardium}$=0.9±0.3).

Ex Vivo Biodistribution Study ^{68}Ga-BADED-2 and ^{68}Ga-BAPDMEN-2 both showed high myocardial SUV and low liver uptake in PET imaging. Therefore, they were selected for *ex vivo* organ biodistribution studies. Figure 6 shows the biodistribution data of these two tracers compared to ^{99m}Tc-sestamibi and ^{18}F-flurpiridaz.

Sixty minutes after injection, all investigated tracers showed high heart uptake and very low blood levels (Fig. 6). ^{68}Ga-BAPDMEN-2 and ^{99m}Tc-sestamibi exhibited the highest heart-to-blood ratio (Tab. 2), indicating the best accumulation in intact cardiomyocytes. Liver and spleen uptake was markedly higher for ^{99m}Tc-sestamibi compared to the ^{68}Ga-Schiff base complexes (Table 2). All tracers show similar lung, kidney, and muscle uptake.

Discussion

The aim of the study was to analyze the structure–property relationship of ^{68}Ga-labeled Schiff base derivatives for their applicability as myocardial PET tracers. These tracers were not aimed as pure myocardial perfusion tracers but as indicators of a myocardial cell damage. The tracers should be taken up into the myocardial cell and retained their as long as the negative mitochondrial potential persists. In damaged myocardial areas, *e.g.*, by ischemia, not only the tracer supply by perfusion will be reduced but also intracellular retention. For this reason, the injured region will show a lower tracer accumulation in the PET image in contrast to the surrounding intact myocardium which allows localization of the damage.

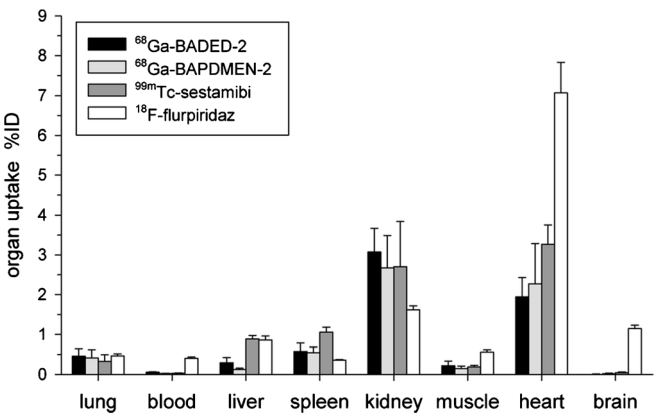

Fig. 6. *Ex vivo* biodistribution data of ^{68}Ga-BADED-2, ^{68}Ga-BAPDMEN-2, ^{99m}Tc-sestamibi, and ^{18}F-flurpiridaz. Values are expressed as percentage of the injected dose per gram tissue at 1 h p.i. (n=4).

The used derivatives varied in their substitution pattern, containing (bromine-, alkoxy-, nitro-, alkyl-, and *N*-ethyl-groups) and formed ^{68}Ga-Schiff base complexes with different molecular structures and physicochemical properties. The stability in isotonic saline, human serum, or apotransferrin solution was above 92 % for almost all tracers studied independently from the backbone or the substitutes introduced. Only ^{68}Ga-BAPEN-3 and ^{68}Ga-BAPEN-2 showed markedly lower values, especially in an apotransferrin solution (Fig. 3). Obviously, in these compounds, the Ga^{3+} seems less strongly complexed. Both tracers are based on the same backbone (BAPEN). The analogous tracers ^{68}Ga-BADED-3 and ^{68}Ga-BADED-2 (carrying the same substitutes) based on a backbone (BADED), which is rather similar to BAPEN, were quite stable. It can be concluded that the backbone is essential for the stability. But also the substitutes play a role since ^{68}Ga-BADED-1 based on the same backbone but carrying substitutes with two methoxy-groups complexed the Ga^{3+} rather strong (Fig. 3).

The ability of a radiopharmaceutical to cross the cell membrane depends mainly on the lipophilicity. Piwnica-Worms *et al*. described a direct correlation between lipophilicity and myocardial uptake of different ^{99m}Tc(I)-radiotracers [21]. Analogue to this, it was expected that the more lipophilic the ^{68}Ga-Schiff base complex, the more it may be able to cross the cell membrane and accumulate in the cells. On the other hand, high lipophilicity might retain the tracer in the lipid layer of the cell membrane instead of entering the intracellular compartment. Previous studies on ^{99m}Tc-tracers revealed that an optimum logP value for myocardial imaging might be 0.5–1.5 [22, 23]. The authors proposed that logP<0.5 show insufficient cell uptake whereas logP>1.5 leads to high protein binding and a slow clearance [23].

The aim of the present study was to systematically modify the structure and by this the physicochemical properties (*e.g.*, lipophilicity) of the ^{68}Ga-Schiff bases and to quantify its impact on *in vitro*, *ex vivo*, and *in vivo* distribution behavior. The synthesized ^{68}Ga-Schiff base complexes show a broad spectrum of logD values ranging from 0.87 to 2.72. The measurements reflect the initial assumption that methoxy-, ethoxy-, and especially bromine-groups increase lipophilicity most. The highest logD value was obtained in the compound where a methoxy- and a bromine-group were combined (BADED-8).

The complexes were quite divergently accumulated in isolated HL-1 cardiomyocytes (Fig. 4a). However, the cellular uptake was only slightly correlated with the lipophilicity of the compound (r=0.296), which is in agreement with the results of Tsang *et al*. who described *ex vivo* biodistribution experiments in rats that ^{68}Ga-Schiff base complexes with logP>1.0 did not have a higher myocardial uptake than those with lower logP values [14]. Additionally, it was observed that small changes in structure lead to significant changes in biodistribution, which was also seen in the present results.

Table 2. Heart-to-organ ratio of the concentrations of ^{68}Ga-BADED-2, ^{68}Ga-BAPDMEN-2, ^{99m}Tc-sestamibi, and ^{18}F-flurpiridaz at 60 min p.i. (n=4)

Compound	Heart:blood	Heart:lung	Heart:liver
^{68}Ga-BADED-2	35.73±4.25	4.22±1.66	6.64±2.15
^{68}Ga-BAPDMEN-2	113.93±9.59	5.48±2.28	18.61±3.67
^{99m}Tc-sestamibi	109.40±6.95	10.04±2.57	3.69±0.95
^{18}F-flurpiridaz	17.52±1.83	15.21±1.92	8.20±1.35

Impact of Substitutes

When comparing solely the results of compounds with the same backbone (BADED) carrying different substitutes, it becomes obvious that the cellular uptake (without valinomycin) is almost independent from the lipophilicity. Tracers with bromine-groups (BADED-3, BADED-8) exhibiting the highest logD values showed only moderate uptake, whereas compounds with *N*-ethyl- or $C(CH_3)_3$-groups (BADED-7, BADED-9) were strongly accumulated in isolated cardiomyocytes even though the *N*-ethyl-group is rather polar. For use as a tracer of myocardial damage, the uptake-ratio in the presence and absence of valinomycin is essential. Here, the compound carrying either the polar *N*-ethyl-group or two moderately lipophilic methoxy-groups showed the lowest ratio. Obviously, both moderate lipophilic in combination with polar substitutes may be of advantage. In the *in vivo* situation, the results were completely different. The tracers with polar substitutes (BADED-7) showed rather low SUV whereas the compounds with a methoxy- or the combination of a methoxy- and a bromine-group showed highest values.

Impact of Backbone

When comparing tracers with the same substitutes but differing backbone, the sole impact of the backbone structure can be obtained. From the *in vitro* experiments, it becomes obvious that the backbone is of high importance for cellular uptake. Independently from the substitutes, BADED showed the highest uptake which was most strongly inhibitable by valinomycin. The results for BAPDMEN and BAPEN were markedly lower indicating that BADED seems to be favorable which might result from the additional methyl groups in the backbone structure. However, once again, the *in vitro* results were not transferable to the *in vivo* situation. Myocardial SUV was only slightly dependent on the backbone but much more on the substitutes. The tracers carrying one methoxy-group at position 3 showed the highest SUV either with the BADED or the BAPDMEN backbone. Obviously, the distribution/elimination in the body which depends on the substitutes is of greater importance than the backbone responsible for uptake in isolated cardiomyocytes. The results reveal that tracers with the lowest lipophilicity showed the highest SUV.

Correlation of In Vitro and In Vivo Results

In order to use PET imaging for the identification of myocardial damage, the tracers should reflect the viability not only by the passive cellular uptake in well-perfused tissue regions but also by a strong accumulation of cationic tracers in intact cells. Although the potential-driven accumulation was seen for all ^{68}Ga-Schiff base complexes, a negative correlation was found

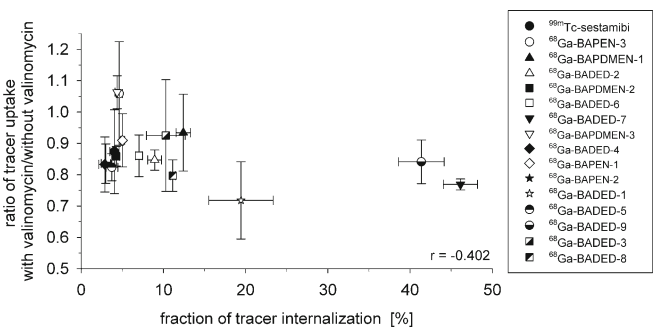

Fig. 7. Correlation of the cellular uptake and the impact of membrane potential for the intracellular accumulation (indicated by the uptake ratio in the presence or absence of valinomycin).

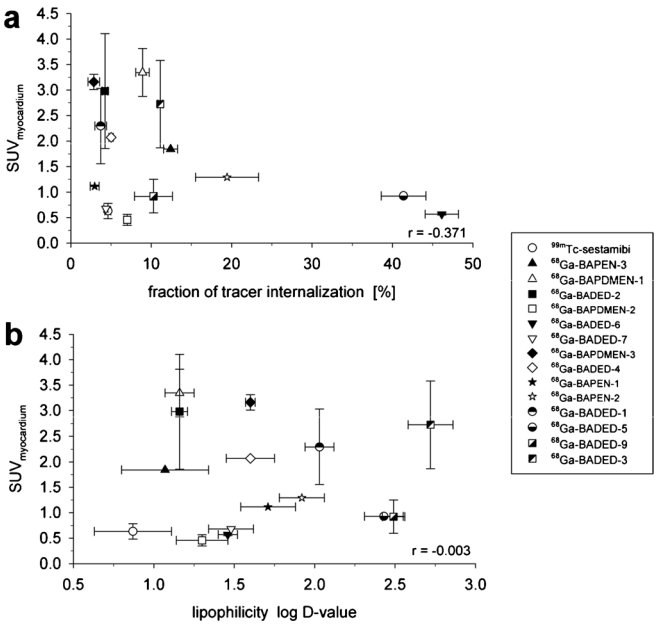

Fig. 8. Correlation of **a** the cellular accumulation determined *in vitro* and **b** the lipophilicity of the tracer (expressed by their logD value) with the standardized uptake values (*SUV*) of the myocardium of the different tracers.

between the passive uptake and the loss of retention when the cells were treated with valinomycin (Fig. 7). These results indicate that for tracers which showed the highest uptake in intact cells, the accumulation was less dependent on the membrane potential but on other properties (*e.g.*, enrichment in the lipophilic cell membrane).

When comparing the cellular uptake data obtained *in vitro* with the standardized uptake values (SUV) from the PET experiments *in vivo*, a clear negative correlation (r=-0.371) was found (Fig. 8a). ^{68}Ga-BADED-7 and ^{68}Ga-BADED-9 which exhibited the highest cellular uptake *in vitro* showed SUV values below 1. The *in vitro* cell uptake values did not reflect the *in vivo* SUV results. One possible explanation could be that due to strong unspecific binding of these tracers to the membrane of blood cells, the tracers do not reach the cardiomyocytes in the *in vivo* setting. In contrast to the results of Tsang *et al*. [14], the myocardial SUV *in vivo* did not correlate with the lipophilicity (Fig. 8b).

Applicability of Tracers

A suitable myocardial tracer should be cleared rapidly from nontarget tissue like lung, blood, and liver. Especially a fast clearance from the abdomen is favored because it might affect the image quality. Besides the heart, also other tissues like the salivary glands, liver, and kidneys which are rich of mitochondria [24] can accumulate cationic tracers. It is also well known that several ^{99m}Tc-radiopharmaceuticals (*e.g.*, ^{99m}Tc-sestamibi) as well as several ^{68}Ga-Schiff base complexes are a substrate of the *P*-glycoprotein (product of the MDR1 gene) [17] and multidrug resistance associated proteins (MRPs). These proteins are expressed in organs of excretion like liver and kidney, which might be the reason for rapid clearance of some ^{68}Ga-Schiff base complexes from liver. Additionally, Kim *et al*. demonstrated that attaching ether groups to cationic ^{99m}Tc-radiopharmaceuticals minimized the liver uptake [23]. This behavior reduces cross-contamination of liver signals to the inferior wall of the myocardium. Similar behavior was seen with ^{68}Ga-Schiff base complexes in the present study. A substitution at the 3-position with a methoxy group (^{68}Ga-BADED-2 and ^{68}Ga-BAPDMEN-2) lead to a prominent uptake or retention in the heart tissue (Fig. 5c) and favorable heart-to-liver ratio (Table 2) as compared to ^{99m}Tc-sestamibi.

When comparing the quality of the new tracers (especially ^{68}Ga-BAPDMEN-2) with the clinically well-established SPECT tracer ^{99m}Tc-sestamibi, several aspects have to be taken into account. As shown in Table 2, the uptake of both tracers from the blood was comparable. However, the ^{68}Ga-based complex showed a four times higher heart-to-liver ratio allowing a better separation of these two tissues. In principle, using PET offers a higher spatial resolution than SPECT which, however, depends on the isotopes used. With ^{18}F, the resolution of PET is approximately three times higher [2]. Due to the higher energy of ^{68}Ga, the spatial resolution of these tracers will be lower [25] but still better than SPECT. Finally, the detection efficiency (sensitivity) of PET is much higher than for SPECT [2] leading to a better detection of small pathologically altered regions. Taking all these aspects together, the new ^{68}Ga-based complexes seems to be a good alternative to ^{99m}Tc-sestamibi SPECT with a higher resolution and sensitivity as well as a better contrast to other surrounding tissues.

Conclusion

The present study clearly demonstrates that ^{68}Ga-Schiff base complexes are suitable for imaging the vital myocardium by PET. The accumulation of the tracer reflects both the perfusion of the myocardium but also the viability of the cardiomyocytes. A local reduction in tracer activity therefore indicates either reduction in tissue perfusion or a cellular damage (however, in most cases, both pathomechanisms are closely correlated). The results of the present study could not describe a clear correlation between specific substitutes and increased tracer accumulation *in vivo*. However, some consistent patterns were observed such as the introduction of methoxy groups in 3-position enhances myocardial tracer accumulation. Following this route, maybe tracer can be developed which show even better uptake *in vivo*. The data also indicate that the results from *in vitro* experiments or the lipophilicity measurements do not predict the suitability in the *in vivo* situation.

In the *in vitro* experiments, it was shown that mitochondrial damage affects the accumulation of several of the tracers. Since ischemia also leads to a reduction in respiratory chain activity, it may be expected that tissue hypoxia also leads to a reduced tracer uptake. However, whether the ^{68}Ga-Schiff based tracers are suitable for the identification of hypoxic myocardial regions has to be addressed in further studies. In parallel, ^{68}Ga-Schiff base amines can be further modified in order to optimize biodistribution behavior. The present results indicate that introducing a methoxy group at the 3-position (*e.g.*, ^{68}Ga-BADED-2 and ^{68}Ga-BAPDMEN-2) seems to favor heart-to-blood (113.9±9.6) and heart-to-liver ratio (18.6±3.7), relevant for good contrast to other surrounding tissues. It can be expected that systematic variation of the chemical structure of the Schiff base backbone or the substitutes further improves relevant pharmacokinetic parameters.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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