DEVELOPMENT OF AN ISATIN-5-SULFONAMIDE BASED PET PROBE FOR IMAGING APOPTOSIS IN VIVO

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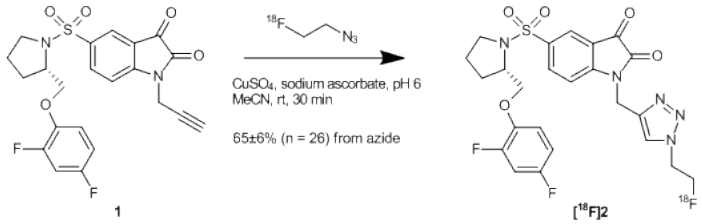
Objectives: Deregulated apoptosis accompanies several diseases including myocardial infarction, ischaemia and neurodegenerative diseases. In contrast, resistance to apoptosis is a hallmark of cancer. It therefore follows that a strategy for selectively imaging apoptotic cell death would be of great value for monitoring the efficacy of novel therapeutics in vivo. and facilitating better patient management.

Methods: Isatin-5-sulfonamides are potent and selective inhibitors of caspase 3/7, the key executioner caspases involved in apoptosis. We evaluated a radiolabeled [^{125}I]isatin and observed rapid in vivo metabolism and clearance. The primary site of metabolic instability was identified and, from a small (n = 12), focused library of isatin-5-sulfonamides a lead compound 2 was identified and radiolabeled (Scheme 1). [^{18}F]2 was evaluated in cell-based and in vivo models of tumor apoptosis using RIF-1 and 38C13 cell lines and also tested for in vivo metabolic stability.



Scheme 1

Results: [^{18}F]2 was prepared using click chemistry in 65±6% (n = 26) radiochemical yield (from [^{18}F]fluoroethyl azide). Cycloaddition was found to be sensitive to temperature, concentration (of 1) and pH. [^{18}F]2 was found to be metabolically stable with a single metabolite at 60 min post-injection in mice as evaluated by radio-HPLC. Uptake of [^{18}F]2 in apoptotic RIF-1 cells was 1.5-fold higher than in control cells and this correlated with a luminescent caspase-3 substrate assay. In RIF-1 tumor bearing mice, a 2-fold increased uptake of [^{18}F]2 was observed in cisplatin treated mice relative to untreated mice. Increased [^{18}F]2 localization by PET imaging was observed in 38C13 tumors in mice treated with cyclophosphamide (compared to control tumors). Apoptosis was confirmed in the drug treated 38C13 tumors by ex vivo immunohistochemistry.

Conclusions: [18F]2 can be radiolabeled in high yield and has good metabolic stability. Utility of [18F]2 as a marker of tumor apoptosis was demonstrated in vivo. [18F]2, therefore, warrants further in vivo validation in tumor models of apoptosis.

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References: G. Smith et al, J Med Chem, 2008, 51 (24), 8057-8067

MICROPET IMAGING OF APOPTOSIS USING RADIOLABELED PEPTIDES

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Objectives: Apoptosis (programmed cell death) is representative biological cell death mechanism. Apoptosis also plays an important role in the regulation of normal development. If we know easily whether apoptosis increases right after anticancer therapy, the successfulness of chemotherapy can be evaluated at early state of cancer therapy and more effective personalized chemotherapy might be chosen. And the survival of transplated stem cell may also evaluated easily by assessment of apoptosis of the cells. Herein, we report effective in vivo imaging of apoptosis using radio-iodinated apoptosis-specific peptide.

Methods: A 6-mer apoptosis-specific peptide, which was screened by the phage display method, was synthesized by peptide synthesizer. One additional tyrosine residue was conjugated at the N-terminal of the peptide for radioiodination. The peptide was radiolabeled with I-131 ([^{131}I]AopPep) and I-124 ([^{124}I]AopPep). For xenogfrafted tumor model, human lung cancer cells (H460) were implanted on each flank in Balb/c nude mice. The tumor was allowed to grow up to approximately 5 mm. One group was treated with 20mg/kg of doxorubicin 3 times every other day in order to induce apoptosis. The mouse was injected with [^{131}I] AopPep or [^{124}I]AopPep, and gamma camera or microPET scan was performed, respectively. For comparison studies, the same mice were also imaged with [^{18}F]FDG and [^{18}F]FLT before [^{124}I]AopPep imaging.

Results: In apoptosis-induced model by doxorubicin, [^{124}I]ApoPep clearly showed higher uptake of activity in tumor region compared with that of non-treated tumor model. However, the tumor region of treated model showed less uptake of [^{18}F]FLT compared to that of non-treated tumor model because of less proliferation activity in treated model. [^{18}F]FDG did not show any significant uptake difference between treated and non-treated group.

Conclusions: The Apoptosis-specific peptide was prepared and radiolabeled by I-131 and I-124 successfully. MicroPET studies clearly showed that the apoptosis induced by chemotherapy could be detected non-invasively by aid of apoptosis-specific peptide.

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NONINVASIVE IMAGING OF DEAD AND DYING CELLS BY NOVEL ORGANO ARSENICALS

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Objectives: The aim of the study was to image dead and dying cells in vivo using functionalized organo arsenicals corroborated with in vitro studies.

Methods: Tripeptide organic arsenical 4-(N-(S-glutathionylacetyl)amino)p-phenyl arsene oxide (GSAO, positive marker) and the corresponding acid (GSCA, negative control) were functionalized with DTPA, DOTA, Cy5.5 and pyrazine using standard conjugation techniques. DTPA derivatives were labeled with $^{111}InCl_3$. For in vitro studies, cultured Jurkat A3 cells were treated with100 ng/mL Fas-Ab either at 4 or 24 hrs and were stained with 10 μM GSAO-pyrazine or GSCA-pyrazine for flow cytometry analysis (Becton Dickinson). For in vitro cell binding assays, Jurkat A3 cells were incubated with either staurosporine or Fas antibody to induce cell death, followed by incubation with radiolabeled compounds, washed and counted for cell associated radioactivity. For in vivo imaging studies, Lewis Lung carcinoma (LLC) cells were implanted in C57BL/6 mice that were treated with cyclophosphamide (CPP, 150mg/kg) on days 10, 11 and 12 post implantation. Both treated and untreated mice were imaged with ^{111}in-DTPA-GSAO, ^{111}in-DTPA-GSCA and ^{99m}Tc Annexin V.

Results: Both GSAO and GSCA functionalization with chelating agents DTPA, DOTA, pyrazine and cy5.5 gave high yields of the conjugated products. Treatment of Jurkat cells with staurosporin or Fas antibody induced both apoptosis and necrosis between 4-20 hrs. GSAO-pyrazine picked up more of late apoptotic cells and necrotic cells at 20 hrs of Stuarosporin or Fas treatment which corroborates well with Annexin V staining of cells. The negative control GSCA-pyrazine did not show significant uptake in all conditions. Studies were also conducted with ^{111}In-DTPA-GSAO and ^{111}In-DTPA-GSCA on Jurkat cells treated with Staurosporin and Fas. The uptake ratio of treated vs. untreated dead and dying cells for ^{111}In-DTPA-GSAO was 6.5, 4.00 and 4.00 when the cell death was induced by 2 ng/mL Fas (20 hrs), 0.08 uM staourosporine (20 hrs) and 100 ng/mL Fas (4 hrs) respectively. In contrast, ^{111}In-DTPA-GSCA showed a ratio 1.5. In the whole body imaging of cell death using SPECT-CT, CPP treated mice bearing LLC tumor showed an increase in uptake of ^{111}In-DTPA-GSAO in tumor and kidney compared to the untreated mice (Fig. 1). ^{99m}Tc-Annexin also showed a higher uptake in tumor, liver and kidney post treatment vs. no treatment. ^{111}In-DTPA-GSCA did not pick up the tumor apart from the little non-specific uptake. On the contrary, ^{99m}Tc-Annexin showed a higher uptake in kidney and not in the tumor post treatment. Thus, radiolabeled GSAO and Annexin V behave differently both in vitro as well as in vivo. However, both pick up dead and dying cells in all models.

Conclusions: GSAO is a novel tripeptide organic arsenical which targets dead and dying cells both in vitro and in vivo and exhibits more favorable biodistribution than Annexin V in C57BL6 mice bearing LLC tumor treated with CPP.

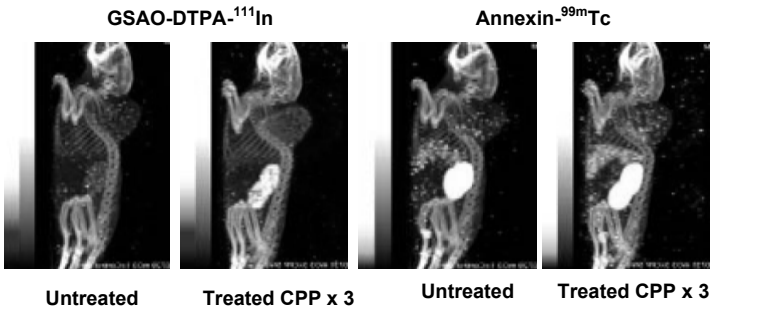

Fig 1: Uptake of GSAO-DTPA-^{111}In and, ^{99m}Tc Annexin V in CPP treated mice:

18F-LABELED ISATIN AS PUTATIVE BIOMARKER FOR THE SPECIFIC IMAGING OF ACTIVATED CASPASES IN APOPTOSIS

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Objectives: A dysregulation of apoptosis occurs in many diseases such as in the clinical scenarios of myocardial infarction, stroke and cancer. The development of tracers for the specific imaging of apoptosis is therefore of utmost scientific and clinical interest. Caspases, an enzyme class playing a decisive role in the execution of apoptosis, represent a biological target to directly diagnose apoptosis-associated diseases. We and others propose non-peptide isatin-based caspase inhibitors as radioligands for the non-invasive visualization of activated effector caspases in apoptosis [1-3].

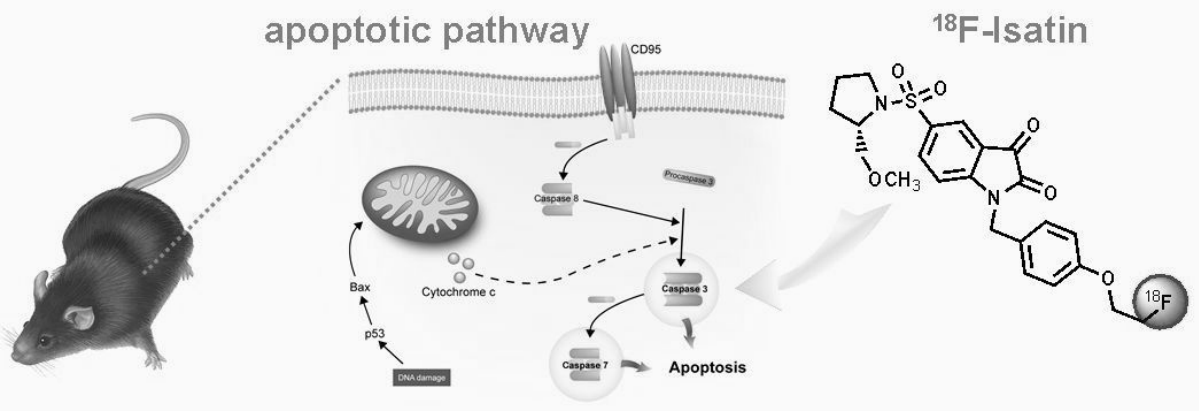

Fig. 1 (S)-1-(4-(2-[^{18}F]Fluoroethoxy)benzyl)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (^{18}F-Isatin) reaching the activated caspases-3 and -7.

Methods: We evaluated the caspase inhibition potency of the non-radioactive counterpart of the model tracer ^{18}F-Isatin by in vitro fluorogenic assays and cellular apoptosis assays. Cellular apoptosis assays were performed in human apoptotic endothelial cells (ECs). Preliminary digital ex vivo microautoradiography (μ-Imager) as well as small-animal PET (quadHIDAC) were realized to evaluate the potential of ^{18}F-Isatin to function as biomarker for the specific imaging of activated caspases in mouse models of ischemia/reperfusion (I/R) injury, i.e. myocardial I/R and middle cerebral artery occlusion (MCAO) model.

Results: Non-radioactive F-Isatin shows a high caspase-3 (IC_{50} = 36 nM) and -7 (IC_{50} = 93 nM) inhibition potency in vitro. Western blot analysis of caspase-3 in apoptotic ECs indicates that caspase processing is inhibited by F-Isatin beginning at concentrations of 10 μM. After i.v. injection of ^{18}F-Isatin into the myocardial I/R model (45 min ischemia / 60 min reperfusion, ID 100 MBq) ex vivo autoradiography of 40 μm cryo-sections of the heart ventricles indicated an increased tracer-associated signal in the area-at-risk 120 min p.i. compared with the non-injured myocardium. TUNEL assays and caspase-3 immunohistochemistry verify the presence of apoptotic cells. Predosing using a 100-fold excess of F-Isatin suppresses the tracer-associated signal in the affected heart tissue. Preliminary in vivo experiments using small-animal PET and the MCAO model (30 min ischemia / 16 h reperfusion, ID 10 MBq) showed a significant tracer-associated signal in the affected brain hemisphere 120 min p.i. of ^{18}F-Isatin.

Conclusions: By introducing our prototype ^{18}F-labeled caspase-targeted radioligand we have shown that isatin-based imaging of activated caspases in apoptotic I/R injured tissues is feasible, a crucial step towards a future new preclinical and clinical imaging approach.

Research Support: This study was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG), collaborative research center SFB 656 MoBil, Muenster, Germany (project A3).

References: [1] Kopka et al. J Med Chem 2006, 49: 6704-15. [2] Zhou D et al. Bioorg Med Chem Lett 2006, 16: 5041-46. [3] Smith G et al. J Med Chem 2008, 51: 8057-67.

18F-LABELED PYRIMIDINE-2,4,6-TRIONES AS POTENTIAL PROBES FOR IMAGING ACTIVATED MMPS

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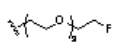
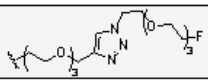
Objectives: Pathological expression and activation of matrix metalloproteinases (MMPs) is associated with cancer, atherosclerosis, stroke, arthritis, and other diseases. The aim is the development of a non-invasive, in vivo imaging approach to detect upregulated levels of activated MMPs. Potentially, radiolabeled MMP inhibitors (MMPIs) are useful tools for the imaging of activated MMPs. This work presents the syntheses and in vitro evaluation of fluorinated MMPIs based on the pyrimidine-2,4,6-trione lead structure RO 28-2653 and the radiosynthesis of the ^{18}F-labeled derivative 1b as potential tracer for imaging activated MMPs with PET.

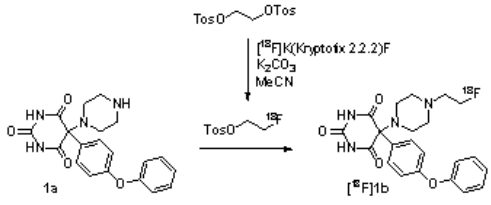
Methods: In previous work we modified the chosen lead structure RO 28-2653 and initiated a radiotracer approach with the goal to facilitate MMP imaging [1-2]. In a further study we evaluated a ^{124}I-labeled model tracer, a derivative of RO 28-2653, that was synthesized and radioiodinated in our laboratories, in the HT-1080 fibrosarcoma xenograft mouse model that is characterized by elevated levels of activated MMPs. Small-animal PET images showed that the tracer candidate slowly accumulates in the tumors 24 h p. i., giving the best tumor-to-background ratios not until 6 days p. i. In this approach we aimed at the development of (radio)fluorinated derivatives with modified pharmacokinetics. Therefore, we synthesized the three fluorinated analogues 1b-d in multistep organic syntheses (Table 1). The MMP inhibition potencies of 1b-d for MMP-2, -8, -9 and -13 were examined in fluorogenic in vitro inhibition assays and expressed as IC_{50} values. The radiosynthesis of [^{18}F]1b, the radiolabeled counterpart of the basic structure 1b, was evaluated and performed in 2 steps starting with precursor 1a.

Table 1: Synthesised pyrimidine-2,4,6-triones **1a-d** with the corresponding calculated log*D*-values.

Cpd.	R	clog*D*[a]
1a	H	2.62
1b		2.88
1c		1.72
1d		0.10

[a] log*D* values were calculated with ACD/ChemSketch Labs 6.00 (log*D* = log*P* at physiological pH)

Figure 1: Radiosynthesis of compound **[^{18}F]1b**.



Results: Fluorinated pyrimidine-2,4,6-triones 1a-d were successfully synthesised in multistep organic syntheses. Compound 1a was obtained in 17% overall chemical yield (3 steps). The N-substituted derivatives 1b-d were yielded in 1% (7 steps), 4% (8 steps) and 0.5% (13 steps), respectively. The synthesized compounds 1a-d are potent inhibitors of MMP-2, -8, -9 and -13 with IC_{50} values in the nanomolar range (37-237 nM). Furthermore, [^{18}F]1b, the first radiofluorinated representative of this kind of MMPIs, was successfully radiolabeled in 2 steps using 1a and the radiosynthon 1-[^{18}F]fluoro-2-tosyloxyethane. After HPLC purification and formulation radiochemical yields of 11.5±0.9% and radiochemical purities of 97.8±0.1% were achieved for the target compound. The synthesis time was 147±29 min from EOB (n=3) (Figure 1).

Conclusions: We identified the 3 fluorinated derivatives of the lead structure RO 28-2653 1b-d as potent MMPIs. Hydrophilic modifications of the piperazine residue (e.g. with mini-polyethylenglycol and 1,2,3-triazole subunits) that occupy the S_2' enzyme pocket were tolerated, giving the possibility to fine-tune the lipophilicity in this series. Moreover a first radiofluorinated model compound ([^{18}F]1b)] was synthesised that will be used for the in vivo evaluation in animal models characterized by elevated levels of activated MMPs using small-animal PET.

Research Support: This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) Sonderforschungsbereich 656 MoBil, Münster, Germany (projects A2 and B1).

References: [1] Kopka et al., J Label Compd Radiopharm. 2007; 50: S397. [2] Breyholz et al., J Med Chem. 2005; 48: 3400-3409.

FLUORINE-18 LABELING AND EVALUATION IN RATS AND TUMOR-BEARING MICE OF THE TENASCIN-C-BINDING APTAMER TTA-01 USING [18F]FPyME

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Objectives: Targeting molecular signatures of a disease is of great interest for diagnosis and therapy, particularly in oncology. Tenascin-C is an extracellular matrix protein that is involved in tumorgenesis which expression levels are elevated in a variety of human tumors. Several antibodies have been labeled to target this protein but results remain unsatisfactory due to the persistence of high blood-radioactivity levels hampering a good signal to noise ratio. TTA-01 is a size-minimized (39 mer, 13.4 kDa), chemically modified RNA aptamer that binds to the fibrinogen-like domain of human Tenascin-C [1]. The 5'-end was derivatized with a hexyl-aminolinker bearing an N2S peptidyl chelating moiety (MAG2) permitting the labeling of the aptamer with radiometals [2,3]. The presence of a free sulfhydryl function also offers an opportunity for its labeling with [^{18}F]FPyME, a [^{18}F]maleimide reagent [4]. The fluorine-18 labeling of TTA-01 as well as in its vivo evaluation in rats and tumor-bearing mice are reported herein.

Methods: [^{18}F]FPyME was prepared using a three-step radiochemical pathway already reported [4]. [^{18}F]FPyME, after HPLC-purification, was conjugated with TTA-01 (0.650-0.850 mg) in 1 mL of a 1/9 (v/v) mixture of DMSO and 0.1 M aq. PBS (pH 7.5) at room temperature for 15 minutes. The product of TTA-01 coupling with [^{18}F]FPyME (c-[^{18}F]TTA-01) was purified by semipreparative reverse-phase HPLC (Waters, μBondapak® C-18). Finally, desalting and formulation of c-[^{18}F]TTA-01 in aq. 0.9% NaCl was performed using a Sephadex NAP-10 cartridge. Whole-body biodistributions and pharmacokinetics of c-[^{18}F]TTA-01 were evaluated in rats and in nu/nu mice bearing U251 human glioblastoma xenografts using our microPET FOCUS 200 tomograph.

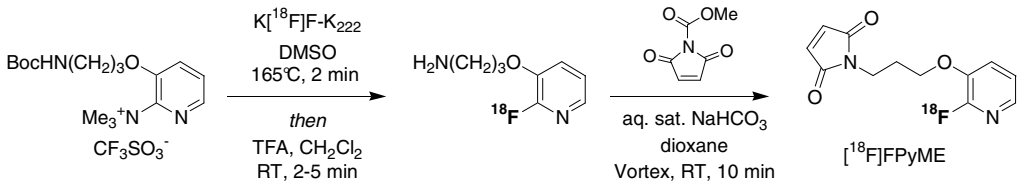
Results: Typically, 5.2-7.5 GBq of radiochemically pure [^{18}F]FPyME could be obtained after HPLC in 110 minutes starting from a cyclotron production batch of 37-51 GBq of [^{18}F]fluoride. Conjugation of [^{18}F]FPyME with TTA-01 was achieved in non-decay-corrected 50% to 70% yields and c-[^{18}F]TTA-01 was easily purified from non-reacted [^{18}F]FPyME using HPLC. Routinely, 0.6-1.4 GBq of HPLC-purified and formulated c-[^{18}F]TTA-01 could be obtained in 65-75 minutes starting from the above-mentioned [^{18}F]FPyME batch with specific radioactivities ranging from 18.5 to 111 GBq/μmol. MicroPET studies showed that in vivo the main routes of elimination of c-[^{18}F]TTA-01 were the urinary and hepato enteric pathways in both rats and mice. In tumor bearing mice, tumor to muscle ratios increased from 3 ± 1 at 60 min to 5.6 ± 0.8 at 90 min (derived from ROIs on microPET images) and to finally 52 ± 25 at 270 min (calculated from organ counting) after radiotracer injection.

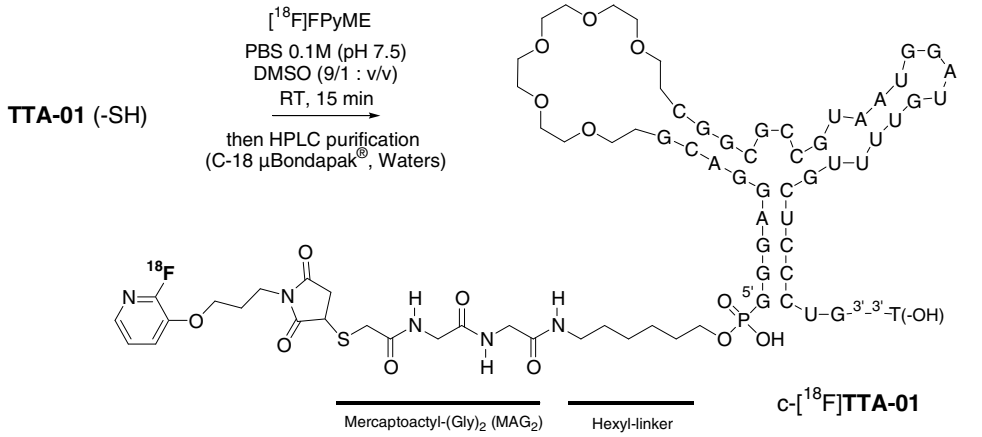
Conclusions: The fluorine-18-labeled reagent [^{18}F]FPyME has been successfully used for the prosthetic labeling of the Tenascin-C-binding aptamer TTA-01. The first applications of this radiofluorinated aptamer are encouraging and may open the possibility of in vivo tumoral characterisation of Tenascin-C in solid tumors.

Research Support: Supported by EMIL (European Molecular Imaging Laboratories) EU contract LSH-2004-503569.

References: [1] Hicke et al. J. Biol. Chem. (2001), 276, 48644-48654. [2] Hicke et al. J. Nucl. Med. (2006), 47, 668-678. [3] Friebe et al. J. Label. Compd. Radiopharm. (2007), 50(S1), S387. [4] de Bruin et al. Bioconj. Chem. (2005), 16, 406-420.

Figure.





RADIOLABELLED MATRIX METALLOPROTEINASE (MMP) INHIBITORS FOR IN VIVO IMAGING OF UNSTABLE PLAQUES USING PET AND SPECT 1

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Objectives: Despite considerable advances in the treatment of cardiovascular disease, it remains the leading cause of mortality and morbidity in the Western world. Frequently, acute clinical events are due to disruptions of lipid-rich unstable plaques. In vivo molecular imaging of these unstable plaques might visualise different stages of the disease (screening, diagnostic and prognostic) and reduce mortality rates, as timely intervention would become feasible. The isoenzymes MMP-2 and MMP-9 within unstable plaques are potential imaging targets, as they are overexpressed. At present, a number of radiotracers are under investigation as imaging ligands of MMP,[2] but most show low subtype selectivity. Here, a new strategy for the synthesis of the selective inhibitors and their radiolabelled analogues is described, which may enable selective non-invasive imaging of vulnerable plaques using SPECT and PET.

Methods: The MMP subtype-selective inhibitors (1a-d) were prepared by reaction of di-tert-butyl protected iminodiacetic acid (IDA) with the appropriate 4-(4-halo-phenoxy)-benzenesulfonyl chloride in the presence of triethylamine, followed by deprotection with formic acid and condensation with hydroxylamine. The IC_{50} values for inhibition of MMP-9, MMP-2, and MMP-1 activities were determined using commercially available assay kits. The ^{123}I-analogue of 1d was obtained from the appropriate stannylated precursor. Biodistribution experiments were performed by i.v. injection of [^{123}I]1d (~6 MBq) to BL-6 mice (6 weeks old, male). At selected time points after dosing, mice were sacrificed by cervical dislocation. under anaesthesia (isoflurane). Tissues of interest were excised and weighed, and radioactivity was measured with a γ-counter.

Results: Overall yields and MMP inhibitory potencies of compounds 1 are given in Table 1. Radioiodination of 1d was performed in 95% decay corrected radiochemical yield and >98% radiochemical purity. Biodistribution experiments showed little de-iodination and normal clearance of the ligand.

Conclusions: A series of highly potent and subtype selective MMP inhibitors has been synthesized and compound 1d was successfully labeled with ^{123}I. Biodistribution studies showed no abnormal clearance of [^{123}I]1d. Imaging and biodistribution studies in APO-E KO mice, a validated model for artherio sclerosis and instable plaques, are ongoing.

References: 1) VA Pinas, et al, Patent application filed P84636EP00 2) MA, Santos, et al, Bioorg Med Chem 14 (22): 7539-50.

Compound		X	Yield (%)	IC_{50}			Selectivity	
				MMP-1 [nM]	MMP-2 [nM]	MMP-9 [nM]	MMP1/ MMP2	MMP1/ MMP9
VP137	1a	H	71	1670 ±980	1.89 ± 1.11	4.31 ±2.06	893	387
	1b	F	58	4690 ±2220	0.13 ± 0.03	0.27±0.15	36076	17370
	1c	Br	73	910±560	0.87 ± 0.40	1.95 ±1.10	1046	466
	1d	I	39	1320 ±790	7.64 ± 1.43	1.61 ±1.41	172	819