Development of PET tracers for investigation of arginase-related pathways

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Arginase is a manganese-containing metalloenzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea. Two arginase isoforms coexist: cytosolic type I (Arg1), predominantly expressed in the liver, and primarily involved in ureagenesis; and mitochondrial type II (Arg2), widely expressed in extrahepatic tissues and mainly involved in the production of L-ornithine outside the urea cycle. L-Ornithine is a precursor of polyamines, proline, and glutamate, which are essential for collagen synthesis, tissue repair, cell proliferation, neuronal development, and in the regulation of immune and inflammatory responses. In parallel, the levels of arginase expression inversely influence the activity of endothelial, neuronal, and inducible nitric oxide synthases (e/n/iNOS), a group of enzymes competing for the same substrate (L-arginine) to catalyze the production of nitric oxide (NO•). The delicate physiological equilibrium between arginase and NOS can be disrupted by oxidative and inflammatory signalling pathways. Arginase overexpression, with the consequent reduction of NO• and increase of polyamines and proline levels, has been associated with a series of pathological processes that range from cardiovascular, immune-mediated, inflammatory and tumourigenic conditions to mental disorders (1). Therefore, arginase is a potential biomarker of disease progression and severity and has been regarded as a possible target for therapy with arginase inhibitors.

As arginase is a potential biomarker of disease and a novel therapeutic target, it was hypothesised that arginase inhibitors could be used as reference scaffolds to develop molecular imaging probes. Among molecular imaging techniques, positron emission tomography (PET) is especially suitable for mapping physiological processes with high sensitivity with small radiolabelled molecules. Using a Cu-mediated ^{18}F-fluorination strategy, radiolabelled arginase inhibitors were synthesised and evaluated in vivo for the first time. The best candidate (^{18}F-FBMARS) was obtained with a radiochemical yield of 4% ± 1% and a molar activity up to 72 GBq.µmol^{-1}. The radiotracer's incubation with arginase expressing cells and asthmatic lung sections showed specific binding that could be blocked (up to 75%) by the pretreatment with arginase inhibitors. Micro PET studies indicated fast blood clearance of the radiotracer (7.3 ± 0.6 min), arginase-mediated uptake, and a tumour accumulation peak nearly 40 minutes after intravenous administration. The obtained results demonstrate the potential use of arginase inhibitors as PET radiotracers for mapping changes in arginase expression that are intrinsically related to poor outcomes, which may aid in the early diagnosis of certain diseases, treatment follow-up, or selection of patients for arginase-inhibiting or arginine-deprivation therapies.

The successful synthesis of a radiolabelled arginase inhibitor opened new perspectives on developing a customised molecular imaging "toolbox" to investigate the possibility of using statins to influence arginase/NOS signalling pathways. In addition to the well-known cholesterol-lowering action (due to inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A, HMG-CoA, reductase), the success of statins became increasingly connected with broader pleiotropic effects. Beyond cardiovascular diseases, statins have been associated with potential protective effects on respiratory, carcinogenic, and neurodegenerative disorders (2). A relatively recent phenomenon that has attracted research interest, and whose mechanisms are still debatable, is whether or not statins have a therapeutic potential in arginase-overexpressing pathological states. Several studies have demonstrated the ability of statins to inhibit arginase and/or increase NO• levels,

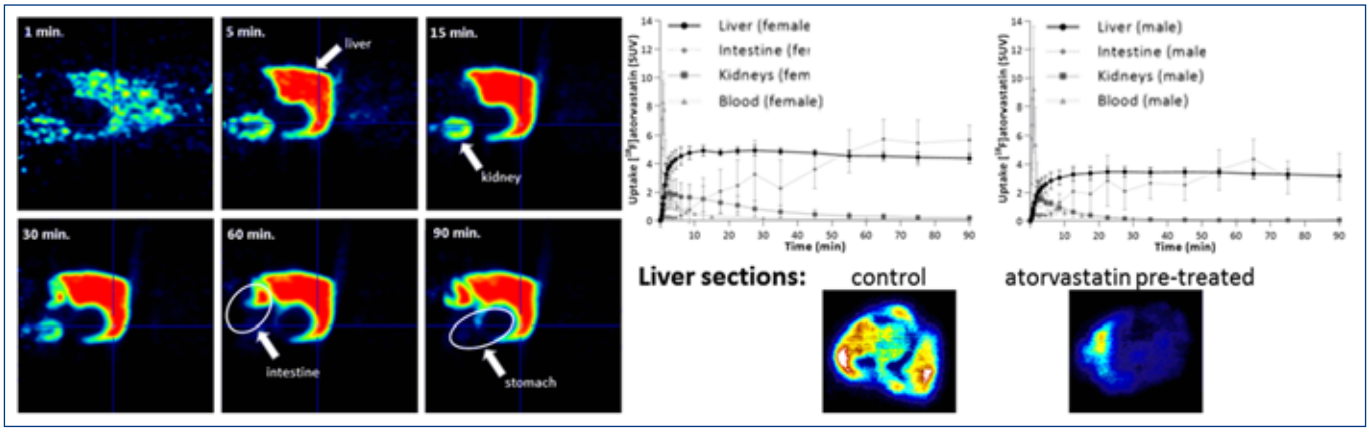

Figure 1. ^{18}F-Atorvastatin: PET projections, time-activity curves, and autoradiography of the liver.

leading to the hypothesis of using this class of drugs for innovative or complementary therapies, especially to suppress tumours or as a treatment for airway remodelling in asthma (3). Atorvastatin, one of the most potent statins in clinical use, seems to have a particular propensity for these additional therapeutic effects. As the exact off-target mechanisms of statins, along with the reason why many patients show resistance or insufficient response to this class of drugs, are still controversial, a radiolabelled analogue of atorvastatin was synthesised to enable access to high-resolution, sensitive, and quantitative nuclear imaging and analytical research techniques.

The synthesis of ^{18}F-atorvastatin was achieved via an optimised version of Ruthenium-intermediated ^{18}F-deoxyfluorination. ^{18}F-atorvastatin was reliably yielded in 23% ± 6%, with a molar activity up to 100 GBq.µmol^{-1}, and radiochemical purity of >99% (4). Preliminary *in vitro* evaluations showed the ability of ^{18}F-atorvastatin to cross the hepatic cell membrane and accumulate in the cytosolic and microsomal fractions, where HMG-CoA reductase is known to be highly expressed. Blocking assays with rat liver sections confirmed the specific binding to this enzyme, and further autoradiography on atherosclerotic rat aorta revealed a significantly higher accumulation (up to 80%) when compared to a healthy reference.

For the *in vivo* determination of biodistribution and kinetics, ^{18}F-atorvastatin was administered in healthy female and male rats. PET imaging studies revealed an extensive liver uptake (figure 1) and did not identify any off-target accumulation site beyond hepatic tissue, which is the primary site of action for HMG-CoA reductase inhibitors. However, the potential pleiotropic effects of statins might be pathology-dependent (e.g. sites of inflammation or cell proliferation), dose-dependent (thus not perceptible with the picomolar amounts administered), or occur as a downstream consequence of liver metabolism, and further studies will be needed to clarify this poorly understood phenomenon.

In summary, the works in this thesis entail the original development, optimisation, and evaluation of radiofluorinated arginase inhibitors and atorvastatin. These results are a first step towards the use of PET to investigate arginase-related pathways and aid further studies to assess the efficacy and underlying mechanisms of the use of statins to alter polyamines and NO• levels.

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