Imaging P-glycoprotein: The gatekeeper of the blood-brain barrier

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6.1 Summary

The blood-brain barrier plays a pivotal role in the regulation of access of compounds to the brain. It limits brain penetration of small molecules by passive diffusion and paracellular passage and, consequently, it protects the CNS from potentially toxic substances. Transcellular passage through the membrane of these endothelial cells is possible by selective transport (mainly from blood to brain) of, for example, amino acids, vitamins and sugars via their specific transporters. Active efflux transporters, driven by ATP, mediate the transport of structurally diverse compounds from brain to blood. The most prevalent ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) 1 have been studied thoroughly over the last few decades.

In **Chapter 1** positron emission tomography (PET) is introduced as an *in vivo* imaging tool to study these ABC transporters. As described in **Chapter 2**, PET has proven to be a powerful method to investigate and measure the function of ABC transporters, and their role in the development of neurodegenerative diseases such as Alzheimer's Disease (AD). It is important that a radiopharmaceutical is specific for P-gp to give reliable PET results. *(R)*-[^{11}C]verapamil is the most widely used P-gp PET tracer for assessing P-gp function and it has shown clinical relevance in AD patients. A disadvantage of *(R)*-[^{11}C]verapamil is the short half-life of 20 min, which limits its use to centres with an on-site cyclotron.

Therefore, two fluorine-18 analogs were synthesized and evaluated in **Chapter 3**. For [^{18}F]**1**, the original [^{11}C]methyl group was replaced by a [^{18}F]fluoroethyl group to simulate the original structure of [^{11}C]verapamil as much as possible. Furthermore, the second analog [^{18}F]**2**, was designed to be less prone to metabolism. Since the removal of the [^{11}C]methyl group is the first metabolic step in the metabolism of [^{11}C]verapamil, the original [^{11}C]methyl group was removed, resulting in a secondary amine. Avoiding this step could lead to a compound that is less prone to metabolism. Since the resulting nor-verapamil is known to also be a substrate of P-gp, the structural change should not interfere with the interaction with P-gp. Next, one of the methoxy groups on the aromatic ring was replaced by a fluoroethyl group, assuming that the stronger C-O bond with respect to the C-N bond would lead to a metabolically more stable tracer.

Both PET tracers showed substrate behavior in tariquidar treated rats with different patterns over time. Whilst [^{18}F]**1** showed higher initial uptake followed by faster washout, [^{18}F]**2** showed slower brain uptake. As tariquidar is an inhibitor of both BCRP and P-gp, the PET study in knockout mice *Mdr1a/b*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$ and WT mice showed that [^{18}F]**2** was more specific for P-gp, despite its unexpected faster rate of metabolism.

In an attempt to circumvent this fast rate of metabolism, deuterated analogs were synthesized and evaluated in **Chapter 4**. Hydrogen atoms in the fluoroethyl groups of [^{18}F]**1** and [^{18}F]**2** were substituted by deuterium atoms to increase the bonds strengths and, consequently, metabolic stability. Furthermore, two additional analogs of [^{18}F]**2** were developed, with a deuterated methyl group bound to the amine, like the original verapamil. The fluoroethyl group of [^{18}F]**3-d$_4$** did not contain deuterium atoms, while the fluoroethyl group of [^{18}F]**3-d$_7$** was deuterated.

Metabolism studies is Wistar rats indeed showed that metabolic stability of [^{18}F]**1** and [^{18}F]**2** improved by inclusion of deuterium in the fluoroethyl group. In addition, increased metabolic stability of the methyl containing analogs, [^{18}F]**3-d$_3$** and [^{18}F]**3-d$_7$** was observed, which may be the result of steric hindrance by the methyl group to execute enzymatic metabolism. [^{18}F]**3-d$_7$** showed the highest stability and was evaluated in *Mdr1a/b$^{(-/-)}$* and WT mice using PET. The similarity in *in vivo* behavior between [^{18}F]**3-d$_7$** and (*R*)-[^{11}C]verapamil, together with improved metabolic stability of [^{18}F]**3-d$_7$**, compared with other fluorine-18 labeled tracers, supports the potential of [^{18}F]**3-d$_7$** as a candidate for clinical translation.

The newly designed potential P-gp PET tracers described in chapters **3** and **4** are substrates of P-gp, which means that their uptake reflects P-gp function rather than expression. A radioactive tracer that directly binds to P-gp is needed to measure P-gp expression, but to date, no successful tracer has been reported yet. Presumed inhibitors show substrate behavior at the low concentrations that are associated with tracer studies. Dose-dependent *in vitro* studies starting at nanomolar concentrations potentially could provide a more reliable prediction for *in vivo* behavior. This approach is investigated in **Chapter 5**.

To improve the predictive value of *in vitro* assays, seven tracers were labeled with tritium and bidirectional substrate transport assays in MDCKII-MDR1 cells were performed at three different concentrations (0.01, 1 and 50 μM) together with inhibition assays using P-gp inhibitors. For comparison, the seven (non-labeled) compounds were used in transport assays in Caco-2 cells at a concentration of 10 μM and in calcein-AM inhibition assays in MDCKII-MDR1 cells. All P-gp substrates were transported in a dose-dependent manner. At the highest concentration (50 μM), P-gp was saturated in a similar way as after treatment with P-gp inhibitors. The best *in vivo* correlation was obtained with the bidirectional transport assay at a concentration of 0.01 μM. It was concluded that a one micromolar concentration in a transport assay or calcein-AM assay alone is not sufficient for correct *in vivo* prediction of substrate P-gp PET ligands. Therefore, putative P-gp inhibitors should be tested at multiple (low) concentrations *in vitro* to predict their *in vivo* behavior.

If a P-gp inhibitor at low nanomolar concentrations could be identified based on the type of studies described above, it could be labeled with a positron emitter and potentially be used as a diagnostic tool to determine expression levels of P-gp and the corresponding state of a neurodegenerative disease. However, this remains a distant prospect as the exact function of P-gp in these diseases still needs to be elucidated. Nevertheless, PET could play a pivotal role in these studies.

6.2 General discussion and Future perspectives

6.2.1 Focus on other ABC transporters

While the development of a P-gp PET tracer is well under way, the other BBB efflux transporters should not be neglected, as their role may be more important than previously thought. There is already a shift towards more research on BCRP, based on the discovery of higher expression levels in humans, compared with P-gp in humans and BCRP expression in rodents.[1] These results were obtained in healthy tissue, but an increasing number of studies address expression levels of BCRP with diseases like AD.[2] However, one should keep in mind that the mode of action of BCRP is different from P-gp, as it forms a homologues dimer in the BBB before it is functional. The literature on expression levels of BCRP does not mention the consideration of the need of two proteins for transport activity.[3] Therefore, one should be critical when interpreting the 1.34-fold higher BCRP expression levels compared with P-gp.[1] More research is needed to elucidate the exact role of BCRP in BBB efflux transport.

PET is an ideal tool to examine the exact function of BCRP. A similar set-up as described in chapters 3 and 4 may be an optimal approach. The success of these studies depends on the specificity of the labeled substrates or inhibitors. At present, no true specific BCRP substrate has been identified yet for *in vivo* studies. A derivative of tariquidar was labeled with carbon-11 and tested in wildtype and knockout animals. Although *in vitro* results showed specific BCRP inhibition with an IC50 of 60 nM, increased brain uptake was detected in both P-gp and BCRP knockout mice, with the highest increase in the triple knockout *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, indicating non-specific behavior *in vivo*.[4]

Another option that might be more viable is labelling of the current BCPR inhibitor Ko143. It showed specific inhibition towards BCRP at low concentrations *in vitro*.[5] The chemical structure of Ko143 gives possibilities for labelling with carbon-11. For a fluorine-18 tracer, the structure would need adaptation.

6.2.2 Multi-target PET tracers

Other recent developments are preclinical studies with multi-target tracers, which interact both with P-gp and BCRP.[6, 7] Is this strategy is only chosen due to the lack of specific BCRP tracers, or really as an added value? The overlap of the substrates, and the co-dependency of the two transporters could rationally lead to one multi-target tracer, but the analysis of these tracers are up to impossible, since too many parameters are added to the equation.

6.2.3 P-gp inhibiting PET tracer

The search for a P-gp expression tracer turned out to be more difficult than thought. For this feature, an inhibitor or binding compound is needed. As mentioned above, it was shown that 'known' inhibitors, such as tariquidar and elacridar, do not act as inhibitors at tracer concentrations, but showed substrate behavior at low (1 nM) concentrations.[8]

In chapter 5, an attempt was made to develop a more reliable *in vitro* test to identify compounds that also act as inhibitor at tracer concentrations. It is clear that a concentration dependent transport assay gives more useful information and therefore it seems to be an essential test prior to the start of *in vivo* studies.

Apart from tracer concentration, other factors may interfere with the straightforward translation from *in vitro* to *in vivo* behavior. For example, one issue is specificity. Tariquidar showed BCRP substrate behavior and therefore no accumulation was observed in the P-gp knockout brain.[9, 10] At high concentrations (>100 nM), however, it is an inhibitor of both ABC transporters. It has been claimed that tariquidar does bind to P-gp at low concentrations and that it is not a substrate, but this is difficult to measure, as BCRP always takes over the transport role.[10, 11]

6.2.4 P-gp expression vs. function

Although in the past, high P-gp expression at the BBB was assumed to be directly related to increased P-gp function, recently, the discussion about this correlation has taken a new turn.[12] Previously, studies on expression and/or function of P-gp in epilepsy models were performed by separate groups using different models and techniques and therefore those studies may not have been comparable. Recently, P-gp expression and function were assessed on an individual animal level and no relationship was found between these two parameters.[13]

Again, to give more insight in this topic, PET could be used as a diagnostic tool with the use of an ^{18}F-labeled P-gp binder. A highly potential binding tracer would be the fluorine-18 labeled isatin compound,[11, 14] that showed a reduction in brain uptake in

P-gp knock-out mice, compared to wildtype. Further studies are needed to confirm this observation, by for example the *in vitro* concentration assays.

6.3 Conclusion

Research continues to increase insight into the role of P-gp and other ABC transporter at the blood-brain barrier. While there is not yet consensus about the importance of P-gp vs. BCRP, the exact mechanisms for substrate and inhibitor behavior, and the relationship between P-gp expression and function, more than ever PET could play an important role to unravel the underlying mechanisms. Using the fluorine-18 PET tracer [^{18}F]**3-d**$_7$, it is now possible to perform more (pre)clinical P-gp studies in a wider range of facilities, even those without a cyclotron.