Function and expression of p-glycoprotein in blood-tissue barriers; translational studies

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Publication date:
2003

Citation for published version (APA):
Bart, J. (2003). *Function and expression of p-glycoprotein in blood-tissue barriers; translational studies*. s.n.

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Chapter 11
Summary and future perspectives

Summary

This thesis reveals additional insights in the functional behavior of P-glycoprotein in the human blood brain barrier, in P-glycoprotein expression and function in animal models, and in P-glycoprotein expression in the human blood-testis barrier and in testicular tumors. The blood-brain barrier is a major obstacle for drug delivery to the brain. Many anti-cancer, anti-epileptic, or anti-HIV drugs reach, due to the bloo-brain barrier function, relatively low brain concentrations, resulting in poor efficacy of these drugs in the brain. For that reason the brain is often considered a sanctuary, harboring unaffected cancer cells during chemotherapy. Increasing drug delivery to the brain may lead to better treatment results, e.g. of brain metastases. One major target to increase drug levels in the brain is the efflux pump P-glycoprotein, which has been demonstrated to efflux many substrates from the brain to the blood. Results of studies in patients, using inhibitors of P-glycoprotein function such as cyclosporin A and PSC 833 showed that treatment with these drugs can lead to neurological side effects. Especially organ transplantation patients using high doses of cyclosporin A as immunosuppressant, are susceptible to develop tremors and epileptic insults. For that reason it is hypothesized that inhibition of P-glycoprotein function may lead to increased levels of cytotoxic P-glycoprotein substrates to the brain. Furthermore it has already been shown in animals that positron emission tomography (PET) with [¹¹C]verapamil offers the opportunity to measure P-glycoprotein function in vivo.

In **Chapter 2** the literature about the blood-brain barrier is reviewed. The blood-brain barrier consists of capillary endothelial cells, closely connected together with tight junctions, and lacking fenestrations. These physicochemical properties withhold the entrance of large and polar molecules in the brain. Lipophilic or small molecules can enter the brain by passive diffusion, which is dependent on their lipid solubility, expressed as octanol water partition coefficient ($\log P_{o/w}$). However, many drugs reach lower brain concentrations than predicted by their $\log P_{o/w}$ and therefore other factors also form the blood-brain barrier. It has been shown that a large category of these drugs are substrate for active, ATP dependent, drug efflux pumps. P-glycoprotein and the multidrug resistance associated protein family (MRP) are well known efflux pumps, but, according to current knowledge,

the wide branched family of human efflux pumps consists of 48 members (http://nutrigene.4t.com/humanabc.htm). P-glycoprotein has a broad substrate specificity and is expressed in many multi-drug resistant (MDR) tumors. P-glycoprotein plays an important role in homeostatic organs such as liver and kidney. In the blood-brain barrier, P-glycoprotein actively transports harmful lipophilic agents from the brain to the blood, contributing to brain homeostasis, which is necessary for a stable micro-environment, enabling proper brain function. P-glycoprotein function can competitively be inhibited by various P-glycoprotein substrates, such as the immunosuppressant cyclosporin A. This P-glycoprotein function can be visualized in vivo with PET and [^{11}C]verapamil.

In **Chapter 3** PET and [^{11}C]verapamil were used (1) to study whether the distribution volume is useful for quantification of (labeled) P-glycoprotein substrate kinetics over the blood-brain barrier and (2) to study how brain distribution volume is affected by P-glycoprotein modulation. We measured the kinetics of the P-glycoprotein substrate [^{11}C]verapamil (0.1 mg/kg) in rat brains using PET and arterial blood sampling. Cyclosporin A was used as P-glycoprotein modulator. [^{11}C]Verapamil kinetics was very well described by distribution volume, computed by non-compartmental Logan analysis. Logan analysis resulted in excellent fits of dynamic PET data, revealing the reversible behavior of [^{11}C]verapamil and its associated distribution volume. The distribution volume in unmodulated rats was 0.65 ml/ml ± 0.23 (mean ± sd). After modulation with 10, 15, 25, 35 and 50 mg/kg cyclosporin A, distribution volume increased to 0.82 ± 0.06, 1.04 ± 0.20, 2.85 ± 0.51, 2.91 ± 0.64 and 3.77 ± 1.23 ml/ml respectively. [^{11}C]Verapamil kinetics was saturable at modulation levels above 25 mg/kg cyclosporin A. The data fitted well by a 4-parameter Hill-plot (r^2=0.79). From this study we conclude that the distribution volume of [^{11}C]verapamil is a valid and potent tool to measure the kinetics of (labeled) P-glycoprotein substrates in vivo at the blood-brain barrier. Brain distribution volume of [^{11}C]verapamil increases dose-dependently by P-glycoprotein modulation.

The quantitative description as developed in the previous chapter was applied to human beings. In **Chapter 4** is described whether the immunosuppressive P-glycoprotein substrates cyclosporin A or tacrolimus can modulate P-glycoprotein function in the human blood-brain barrier. Lung transplantation patients (n=8) using the highest clinical applicable dosages of cyclosporin A or tacrolimus as immunosuppressant, were compared with healthy volunteers (n=10). All individuals underwent a dynamic [^{11}C]verapamil PET procedure. Tissue and plasma kinetics of [^{11}C]verapamil were used to calculate the distribution volume in grey and white matter and the influx constant K$_1$ by means of Logan analysis. With Logan analysis we demonstrated excellent fits of plasma versus brain tissue time-activity curves both in volunteers and in lung transplantation patients. Both

distribution volume of $[^{11}C]$verapamil in the gray matter as well as in white and gray matter together were higher (27 % and 31 %, respectively) in patients than in volunteers (p=0.03), whereas the influx constant K_1 was not affected. Therefore it was concluded that P-glycoprotein function indeed can be modulated at the level of the human blood-brain barrier. The C3435T single nucleotide polymorphism on the MDR1 gene revealed that the CC genotype was for all individuals correlated to a lower distribution volume of $[^{11}C]$verapamil than the CT and the TT genotype (p=0.01).

Chapter 5 describes a human small-cell lung cancer xenograft model in rat brains. In this study the question was posed whether newly formed vessels do express P-glycoprotein, and whether such xenografts do behave different from normal brain tissue with respect to uptake of the P-glycoprotein substrate $[^{11}C]$verapamil. P-glycoprotein was equally expressed in the capillary endothelial cells of normal brain tissue and the xenograft. P-glycoprotein was not expressed on glial cells. No differences were observed between vessel density in normal brain tissue and the xenograft. Without cyclosporin A, uptake of $[^{11}C]$verapamil in the xenograft was 10 times higher than in normal brain tissue (p=0.04). Cyclosporin A increased $[^{11}C]$verapamil uptake in a dose dependent manner in striatum and cortex (p=0.04 and p=0.015). Uptake in cortex and striatum at 50 mg/kg cyclosporin A equals the maximum uptake of $[^{11}C]$verapamil in the tumor which was already reached at 10 mg/kg cyclosporin A (p=0.05). This means that five times less cyclosporin A was required to increase $[^{11}C]$verapamil uptake in GLC4 xenografts to the same level as maximal uptake in normal brain tissue. P-glycoprotein is expressed in newly formed rat vessels, growing into cerebral human small cell lung cancer cell suspension xenografts. The maximal uptake in the xenograft was reached at lower cyclosporin A dose than necessary for maximal $[^{11}C]$verapamil uptake in the normal brain indicating a higher effect on xenografts.

Chapter 6 describes the study performed to elucidate whether the use of the P-glycoprotein substrate $[^{11}C]$carvedilol is of additional value for functional P-glycoprotein imaging with PET. Ideally, for the purpose of brain imaging, tracers should have a lipid solubility, expressed as octanol water partition coefficient (log $P_{o/w}$) between 0.9 and 2.5. The anti-hypertensive β-receptor antagonist carvedilol is a P-glycoprotein substrate with a log $P_{o/w} = 2.0$. It can be labeled with $[^{11}C]$. Cell line experiments in the parental cell line GLC4, and the MDR1 transfected cell line GLC4/P-glycoprotein and the MRP1 overexpressing cell line GLC4/Adr showed that $[^{11}C]$carvedilol uptake increased 3-fold in the P-glycoprotein expressing cell line after pretreatment with cyclosporin A. The parental cell line and the MRP1 overexpressing cell line were not affected by cyclosporin A, nor by the MRP1 inhibitor MK571. Biodistribution studies in rats showed that $[^{11}C]$carvedilol uptake in the brain was increased 5-fold by cyclosporin A. $[^{11}C]$Carvedilol uptake in other

organs was not affected by cyclosporin A. Autoradiopgraphy studies revealed that [^{11}C]carvedilol was homogeneously distributed over the brain and that pretreatment with cyclosporin A increased [^{11}C]carvedilol uptake by maximal 7-fold. Logan analysis of brain kinetics of [^{11}C]carvedilol, obtained from in vivo PET experiments resulted in excellent fits, revealing that [^{11}C]carvedilol is not trapped in the brain. Brain distribution volume of [^{11}C]carvedilol showed a dose-dependent increase of maximal 3-fold after cyclosporin A pretreatment. Above doses of 15 mg/kg there was no change in distribution volume. Compared to [^{11}C]verapamil experiments, described in chapter 2, less cyclosporin A was needed to reach maximal distribution volume, suggesting that [^{11}C]carvedilol is a more sensitive tool to measure P-glycoprotein function in vivo than [^{11}C]verapamil. [^{11}C]carvedilol is a promising tracer to optimize P-glycoprotein imaging with PET.

In **Chapter 7** It has been shown that expression of P-glycoprotein decreases early after brain irradiation. To elucidate whether radiotherapy reduces P-glycoprotein expression and function, right hemispheres of rat brains were irradiated with single doses of 2, 5, 10, 15, and 25 Gy followed by 10 mg/kg cyclosporin A after 5 days, or with a single dose of 15 Gy followed after 5 days by different doses of cyclosporin A (10, 5, and 20 mg/kg), or fractionated irradiation (4 x 5 Gy) and (10 mg/kg) followed by cyclosporin A after 5 days. Cyclosporin A was administered iv 30 min prior to [^{11}C]carvedilol injection. Additionally 4 groups of rats were irradiated with 25 Gy and after 10, 15, 20, and 25 days. These rats were sacrificed, and their brains were removed. Irradiation resulted in a dose- dependent increase on day 5 of [^{11}C]carvedilol uptake up to maximal 20% of the non-irradiated hemisphere. In addition, cyclosporin A increases [^{11}C]carvedilol uptake dose-dependently, in both hemispheres, but [^{11}C]carvedilol uptake was higher (p<0.001) in the irradiated hemisphere. Fractionated irradiation induced the same increase in [^{11}C]carvedilol uptake at the same biological effective dose as single dose irradiation. P-glycoprotein expression decreases between 10 and 20 day after single dose irradiation with 25 Gy, and increases afterwards to the same or higher levels as before irradiation. From these experiments it can be concluded that irradiation of the rat brain induces a reduction of P-glycoprotein expression and function. If this applies to human beings, it can be hypothesized that radiotherapy, which is frequently applied to humans with brain metastases, could be useful to increase delivery of chemotherapy to the brain.

For that reason in **Chapter 8** it is determined in patients whether radiotherapy increases delivery of [^{11}C]verapamil as measured with distribution volume. In this still ongoing study (non) small cell lung cancer patients with brain metastases, treated with palliative whole brain radiotherapy (5 x 4 Gy) undergo a [^{11}C]verapamil PET procedure before and after radiotherapy. Regions of interest are defined on 1) brain metastases, 2) normal brain contralateral of the metastases,

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Chapter 9
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and 3) a large part of normal brain tissue. From these regions [^{11}C]verapamil kinetics in tissue are obtained. During the [^{11}C]verapamil scan blood samples are collected to determine [^{11}C]verapamil kinetics in plasma. Tissue and plasma kinetics are used to calculate the distribution volume and the influx constant K_1 of [^{11}C]verapamil in the different regions. Until now 15 patients entered the study, of which 7 had sufficient condition to undergo the second scan after radiotherapy. All patients were treated with dexamethasone, and if epileptic seizures were present also with fenytoin. Scans were made 14.7 ± 9.3 days (mean ± sd) before and 14.7 ± 5.8 days after the first radiotherapy session. Before radiotherapy mean distribution volume (± sd) in the metastasis is 1.04 ± 0.33 and contralateral of the metastasis 0.60 ± 0.15 (p=0.03). The K_1 was not significantly different (p=0.06). After radiotherapy mean distribution volume and K_1 (± sd) in the metastases were both decreased (p=0.04 and 0.01) in contrast to the contralateral region where distribution volume and K_1 were not affected (p=0.06). Since the distribution volume is linearly related to K_1, changes in P-glycoprotein function in the metastasis, induced by irradiation can not be estimated. Radiotherapy (5 x 4 Gy) does not increase [^{11}C]verapamil delivery to the human brain within two weeks after radiotherapy. However, the initially higher [^{11}C]verapamil delivery to brain metastases is reduced after radiotherapy which can potentially be explained by reduced influx. Therefore, future studies should be performed to elucidate whether radiotherapy should precede chemotherapy or reverse.

Chapter 9
The function of the blood-testis barrier is to protect germ cells against harmful influences, thus also impeding the delivery of chemotherapeutic drugs to the testis. In this sense, the blood-testis barrier shares many properties with the blood-brain barrier, which protects the brain. In this chapter the architecture of the blood-testis barrier is described, and compared to the blood-brain barrier. The blood-testis barrier is a threefold barrier. Firstly a physico-chemical barrier, consisting of continuous capillaries, Sertoli-cells in the tubular wall, connected together with narrow tight junctions, and a myoid cell layer around the seminiferous tubule. Secondly an efflux pump barrier that contains P-glycoprotein in the luminal capillary endothelium and on the myoid cell layer, and Multidrug-Resistance associated Protein 1 (MRP1) located basolaterally on Sertoli-cells. Thirdly the blood-testis barrier consists of an immunological barrier, consisting of Fas-ligand on Sertoli-cells. If the results on imaging and modulation of P-glycoprotein in the brain also apply to the testis, inhibition of P-glycoprotein function potentially offers the opportunity to increase the delivery of cytotoxic drugs to the testis. In the future visualization of the blood-testis barrier function may also be helpful to

determine patient groups, in which testis conservation is safe or to select drugs that are less harmful for fertility.

Chapter 10 The precise localization of P-glycoprotein and MRP1 in the testis and in primary testicular tumors is largely unknown. We studied the localization of P-glycoprotein and MRP1 in the blood-testis barrier in normal testicular tissue and in primary seminomas, non-seminomas, and testicular lymphomas. In normal testicular tissue P-glycoprotein was strongly expressed on myoid cells surrounding the seminiferous tubule, and on Leydig cells and capillary endothelium, but not on Sertoli cells. MRP1 was expressed on Sertoli cells and Leydig cells. Seminomas and non-seminomas expressed P-glycoprotein or MRP1 or both, while lymphomas strongly expressed P-glycoprotein but no or weakly MRP1. Newly formed vessels expressed P-glycoprotein but no MRP1. This was regardless of tumor type. This study illustrates that P-glycoprotein and MRP1 are expressed in different cell layers of the normal testis, which suggests optimal protection of spermatogenesis. The expression of efflux pumps on germ cell tumors may at least partly explain the resistance of these tumors to P-glycoprotein and MRP1 substrates. Both in germ cell tumors and in testicular lymphomas P-glycoprotein expression in newly formed vessels may also contribute to chemoresistance. These findings are in favor of the common practice to remove the affected in case of primary germ cell tumors and testicular lymphomas, irrespective whether the patient already underwent chemotherapy.

Future perspectives

The studies described in this thesis, have provided additional insight in the functional nature of P-glycoprotein in the human blood-brain barrier. From these studies it has become clear that if brain metastases are macroscopically visible, the drug resistance of the metastasis is not solely due to P-glycoprotein function in the blood-brain barrier but also to several other resistance mechanisms present in the tumor. However, microscopic metastases may still be dependent on the normal blood-brain barrier. Therefore, oncology research on P-glycoprotein function in the blood-brain barrier should be directed particularly to the normal blood-brain barrier. [^{11}C]verapamil or [^{11}C]carvedilol PET can be used as a potent tool to determine the capacity of new P-glycoprotein inhibitors, which are currently being developed. Furthermore, if such P-glycoprotein inhibitors are available for clinical applications they should primarily be applied as prophylactic co-treatment with chemotherapy to patients with tumors that metastasize easily to the brain. Future PET studies in patients can be helpful to determine whether patients are eligible for co-treatment with a P-glycoprotein inhibitor. So far, no potent non-toxic inhibitor of P-glycoprotein function is clinically available. Promising newly developed P-

glycoprotein inhibitors, such as ONT-093, LY335979, GF120918, and XR9576 are currently tested in early clinical trials [129,205,207,318,319].

Many patients with other brain diseases, such as epilepsy or neuronal HIV do not respond to brain drugs [159]. It has been suggested in rats and humans that P-glycoprotein in the blood-brain barrier is involved in this resistance [183,264]. Knowledge obtained in this thesis could also be translated to these patient groups to study whether inhibition of P-glycoprotein function in the blood-brain barrier is useful to increase drug delivery the brain. For instance patients with epilepsy, refractory to anti-epileptic drugs could be treated with a new, non-toxic P-glycoprotein inhibitor. Clinical therapy outcome, such as frequency of seizures can easily be compared to brain pharmacokinetics of $[^{11}C]$verapamil measured with PET. Another field off interest is functional imaging of genetic differences. It is known that the MDR1 gene, encoding for P-glycoprotein exhibits several single nucleotide polymorphisms, some of which result in different transport capabilities. A recent study suggests that the single nucleotide polymorphism C3435T on exon 26 of the MDR1 gene, coding for P-glycoprotein, may induce phenotypes with different sensitivity to anti-epileptics [219]. Patients resistant to anti-epileptic drugs were more likely to have the CC genotype. Functional imaging of P-glycoprotein may largely strengthen the conclusions based on the observations in this study. Based on the results of the studies described in this thesis, it is likely that functionally different phenotypes as a consequence of different genotypes can be detected by means of $[^{11}C]$verapamil or $[^{11}C]$carvedilol PET. On the other hand, inconceivable interindividual variances of functional measurements may in some cases be explained by so far unknown differences in genotype.

It is still a matter of debate whether patients with brain metastases can benefit from concurrent radiotherapy and chemotherapy. In our study in rats, treated with irradiation alone, a reduction of P-glycoprotein expression and function was found. This finding justifies studies in patients undergoing radiotherapy, to determine the effects of radiotherapy on brain delivery of P-glycoprotein substrates. Our preliminary study in patients showed after 14 days no effects in normal brain, and a decreased delivery of $[^{11}C]$verapamil to brain metastases. Although this study is ongoing, it is questionable whether macroscopic brain metastases benefit more from chemotherapy following radiotherapy than from radiotherapy or chemotherapy alone. However, other radiation schedules and doses may be subject of future studies to increase drug delivery to the brain.

At present, P-glycoprotein is frequently studied in drug delivery questions. It is currently well known that at least 48 transporters from the ATP binding cassette family contribute to transmembrane transport. Many of these transporters are expressed in the blood-brain barrier, where they are involved in the protection of the brain. However, little is known about the function of these transporters. Many

of these transporters share their substrates. In addition they exhibit different mechanisms of action. It is therefore reasonable that inhibition of one or two of these pumps will only result in a very limited effect on drug delivery to the brain. For that reason future studies should not only investigate P-glycoprotein but also other transporters. Insight in the function these transporters may potentially lead to possibilities to increase drug delivery to sanctuary sites such as the brain and the testis. Additionally, it may also provide knowledge which may lead to increase the protection of the brain or the testis against toxic side effects of harmful agents.