HEALTH SCIENCES

Mikko Gynther

Blood-Brain Barrier Transporters in CNS Drug Delivery

Design and Biological Evaluation of LAT1 and GluT1 –Targeted Prodrugs

Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF EASTERN FINLAND

MIKKO GYNTHER

Blood-Brain Barrier Transporters in CNS Drug Delivery:

Design and Biological Evaluation of LAT1 and GluT1 –Targeted Prodrugs

To be presented by permission of the Faculty of Health Sciences,
University of Eastern Finland for public examination
in auditorium ML3, Medistudia building, University of Eastern Finland, Kuopio,
on Saturday 9th of October 2010, at 12 noon.

Publications of the University of Eastern Finland
Dissertations in Health Sciences
29
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio
2010

Kopijyvä Oy
Kuopio, 2010

Distribution:

Eastern Finland University Library/Sales of Publications
P.O.Box 1627, FI-70211 Kuopio, FINLAND
http://www.uef.fi/kirjasto

ISBN 978-952-61-0212-2
ISBN 978-952-61-0213-9 (PDF)
ISSN 1798-5706
ISSN 1798-5714 (PDF)
ISSNL 1798-5706

Authors address: School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
P.O.Box 1627,
FI-70211 Kuopio, Finland
mikko.gynther@uef.fi

Supervisors: Professor Jarkko Rautio, PhD
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Krista Laine, PhD
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Jarmo Ropponen, PhD
VTT Technical Research Centre of Finland
Espoo, Finland

Reviewers: Professor Marjo Yliperttula, PhD
Faculty of Pharmacy
Division of Biopharmaceutics and Pharmacokinetics
University of Helsinki
Helsinki, Finland

Professor Peter Swaan, PhD
School of Pharmacy
Department of Pharmaceutical Sciences
University of Maryland
Baltimore, USA

Opponent: Jouni Sirviö, PhD
Oy Sauloner Ltd
Kuopio, Finland

Gynther, Mikko. Blood-Brain Barrier Transporters in CNS Drug Delivery: Design and Biological Evaluation of LAT1 and GluT1 –Targeted Prodrugs. Publications of the University of Eastern Finland. Dissertations in Health Sciences 29. 2010. 129 p.

ABSTRACT

The underlying reason for under-penetrated global central nervous system (CNS) drug market is the lack of efficient delivery strategies that enable drugs to circumvent the blood-brain barrier (BBB). Several specific endogenous influx transporters have been identified at the brain capillary endothelium forming the BBB. The aim of the study was to design and synthesize amino acid and glucose prodrugs of ketoprofen and indomethacin and to evaluate their ability to cross the BBB via transporters.

In the present study we were able to demonstrate that ketoprofen-tyrosine and ketoprofen-lysine amide prodrugs are able to cross the BBB carrier-mediatedly. In addition, ketoprofen-lysine amide prodrug was taken up by brain cells *in vivo*, where ketoprofen was released.

In the case of glucose prodrugs the results strongly suggest, that a hydrophilic drug can be attached to D-glucose and still maintain the affinity glucose transporter. However, glucose as a promoiety has several limitations. The stability of ester prodrugs in systemic circulation might not be adequate for clinical use of ester prodrugs for drug brain targeting.

In conclusion, both LAT1 and GluT1 are able to deliver prodrugs across the rat BBB and the parent drug is released in the brain parenchyma. However, more effort has to be done before this prodrug approach is fully applicable for clinical use. Glucose prodrugs are too labile and therefore are not useful to enhance brain uptake. Amino acid prodrugs are more interesting, because there are more options which bond is used between the parent drug and the promoiety. The application of amino acid prodrugs for enhanced brain drug delivery might be limited to small molecular weight drugs with extremely low or non-existent brain uptake.

National Library of Medicine Classification: QV 785, WL 200, QV 76.5, WL 300, QU 55.2, QU 120

Medical Subject Headings: Drug Delivery Systems; Central Nervous System; Prodrugs; Blood-Brain Barrier; Membrane Transport Proteins; Carrier Proteins; Large Neutral Amino Acid Transporter 1; Glucose Transporter Type 1; Ketoprofen; Indomethacin

Gynther, Mikko. Veri-aivoesteen kuljetusproteiinien hyödyntäminen lääkeaineiden keskushermostokohdentamisessa aihiolääkkeiden avulla. Itä-Suomen yliopiston julkaisuja. Terveystieteiden tiedekunnan väitöskirjat 29. 2010. 129 s.

TIIVISTELMÄ

Yksi tärkeä syy useiden keskushermostosairauksien heikolle vasteelle lääkehoitoon on lääkeaineiden riittämätön pääsy vaikutuspaikkaansa. Tämä johtuu veri-aivoesteestä, joka suojaa tehokkaasti aivojen homeostaasia.

Tässä tutkimuksessa selvitettiin voidaanko aihiolääkkeitä hyödyntää keskushermostokohdentamisessa. Aihiolääkkeet ovat lääkeaineen inaktiivisia johdoksia, jotka vapauttavat lääkeaineen ennen imeytymistä, imeytymisen jälkeen tai tietyssä kohdekudoksessa. Näin voidaan vaikuttaa lääkeaineen kulkeutumiseen elimistössä ilman että vaikutetaan sen kykyyn saada aikaan haluttu lääkevaste. Lääkeaineisiin liitettiin biologisesti hajoavilla sidoksella erilaisia aihio-osia. Tavoitteena oli selvittää voivatko kuljetusproteiinit kuljettaa keskushermostoon muodostetun aihiolääkkeen.

Aihiolääkkeiden kyky hyödyntää kuljetusproteiineja tutkittiin eläinkokeiden avulla. Veriaivoesteen läpäisyn lisäksi on tärkeää selvittää kuinka suuri osuus aihiolääkkeestä ja lääkeaineesta on sitoutunut epäspesifisesti aivokudokseen. Molekyylien jakautuminen aivoissa määritettiin yhdistämällä eläinkokeiden ja aivohomogenaateissa tehtyjen *in vitro* -kokeiden avulla.

Väitöskirjatyössä saavutettujen tulosten perusteella voidaan todeta että aihiolääkkeitä voidaan hyödyntää keskushermostokohdentamiseen kuljetusproteiinien avulla. Lisäksi tutkimuksessa pystyttiin osoittamaan, että aihio-osalla voidaan vaikuttaa lääkeaineen jakautumiseen aivojen solunsisäisen ja solunulkoisen nesteen välillä. Tutkimustuloksia voidaan hyödyntää pyrittäessä edistämään erittäin huonosti keskushermostoon kulkeutuvien lääkeaineiden pääsyä vaikutuspaikkaansa.

Yleinen suomalainen asiasanasto: lääkeaineet; aihiolääkkeet; keskushermosto; kohdentaminen

Acknowledgements

The present work was carried out in School of Pharmacy, University of Eastern Finland, during the years 2004-2010. The study was financially supported by the Graduate School in Pharmaceutical Research, the Academy of Finland, the Finnish Funding Agency for Technology and Innovation, the Finnish Parkinson Society, Kuopio University Foundation, Emil Aaltonen Foundation and the Finnish Cultural Foundation, North Savo regional fund.

I am very grateful to my principal supervisor Prof. Jarkko Rautio for introducing me to the field of the prodrug research; I thank you for your support and guidance during my Ph.D. studies. I also thank my other supervisors Dr. Krista Laine and Dr. Jarmo Ropponen for their invaluable advices and help concerning this work. I want to thank all my co-authors: Prof. Markus Forsberg, M.Sc. Aaro Jalkanen, M.Sc. Marko Lehtonen, Dr. Anne Mannila, M.Sc. Lauri Peura, Dr. Jukka Leppänen, Prof. Tomi Järvinen, Dr. Jouko Savolainen, Dr. Tapio Nevalainen, M.Sc. Paula Haapakoski and M.Sc. Johanna Knuuti. I also wish to thank Mrs. Anne Kaikko, Mrs. Katja Hötti, Mrs. Miia Reponen and Mrs. Tiina Koivunen for their help in the laboratory. I owe deepest gratitude to Ms. Helly Rissanen, for her excellent technical assistance in the laboratory.

I am very grateful to Dr. Jouni Sirviö, who kindly accepted the invitation to serve as an opponent of the public examination of dissertation. I express my deepest gratitude to Prof. Marjo Yliperttula and Prof. Peter Swaan for reviewing this thesis and Dr. Ewen MacDonald for revising the language. Prof. Jukka Mönkkönen, the Dean of the Faculty of Health Sciences, and current and former Heads of the Department, Prof. Seppo Lapinjoki, Prof. Antti Poso and Prof. Tomi Järvinen are greatly

acknowledged for providing excellent facilities to work. I wish to thank all my colleagues and friends in University of Eastern Finland for creating such a great working environment.
Finally, my warmest thanks go to my close friends, brother and parents for their endless love and support.

Kuopio, Finland June 2010
Mikko Gynther

List of original publications

The present doctoral dissertation is based on the following original publications:

I Gynther M, Laine K, Ropponen J, Mannila A, Savolainen J, Nevalainen T, Järvinen T, Rautio J: Large neutral amino acid transporter enables brain drug delivery via prodrugs. Journal of Medicinal Chemistry, 2008, 51, 932-936.

II Gynther M, Ropponen J, Laine K, Leppänen J, Haapakoski P, Järvinen T, Rautio J: Glucose promoiety enables glucose transporter-mediated brain uptake of ketoprofen and indomethacin prodrugs in rats. Journal of Medicinal Chemistry, 2009, 52, 3348–3353.

III Gynther M, Laine K, Ropponen J, Leppänen J, Lehtonen M, Knuuti J, Jalkanen A, Forsberg M, Rautio J: Brain uptake of ketoprofen-lysine prodrug in rats. International Journal of Pharmaceutics, 399: 121-128, 2010.

Contents

Abbreviations

ATP	adenosine triphosphate
AUC	area under the concentration curve
BBB	blood-brain barrier
BCSFB	blood-cerebrospinal fluid barrier
CL	clearance
CLogP	calculated partition coefficient
C_{max}	maximum concentration
CSF	cerebrospinal fluid
CNS	central nervous system
CNT2	adenosine transporter
COX	cyclooxygenase enzyme
CSF	cerebral spinal fluid
DNA	deoxyribonucleic acid
ECF	extracellular fluid
$f_{u\ brain}$	the free fraction of the drug in brain tissue
GluT1	glucose transporter type 1
HPLC	high performance liquid chromatography
ICF	intracellular fluid
K_{in}	influx clearance
K_m	the Michaelis constant
K_p	concentration ratio between brain and blood
$K_{p,\ free}$	the unbound brain to plasma concentration ratio
$K_{p,free,cell}$	the concentration ratio between ICF and ECF
LAT1	large neutral amino acid transporter type 1
LAT2	large neutral amino acid transporter type 2
LDL	low-density lipoprotein
Log D	apparent partition coefficent
MAb	monoclonal antibody
MCT1	monocarboxylic acid transporter 1
MDCK	Madin-Darby canine kidney cells
MDR-1	multi-drug resistance 1
mRNA	messenger ribonucleic acid

MRP	multidrug resistance associated protein
NSAID	non-steroidal anti-inflammatory drug
PA	permeability–surface area
P-gp	P-glycoprotein
RAP	receptor-associated protein
SD	standard deviation
SEM	standard error of mean
SPE	solid phase extraction
$\mathbf{t_{max}}$	time to maximum plasma concentration
$\mathbf{V_{max}}$	the maximum transport velocity
$\mathbf{V_{u,brain}}$	the unbound drug volume of distribution in brain
$\mathbf{V_v}$	vascular volume
$\mathbf{y^+}$	cationic amino acid transporter
%ID/g	the percent of injected dose that reaches the brain

1 Introduction

Due to the aging of the general population, the burden of central nervous system (CNS) diseases continues to increase (Pardridge, 2003). It has been estimated by WHO and the World Bank that in the 21st century the costs associated with CNS disorders in Europe will be over one third of the total disease burden (Olesen et al., 2006). In addition, it has been estimated that one out of every three individuals will experience a CNS condition during their lifetime.

Recent advances in biotechnology and pharmaceutical sciences have greatly expanded the number of drugs that are being developed for the treatment of CNS disorders. Drugs identified through novel discovery techniques often do not consider the pharmacokinetic and pharmaceutical properties of the drug candidates. The reasons for the under-penetrated CNS drug market is the lack of efficient delivery strategies that enable drugs to circumvent the blood-brain barrier (BBB) and poor knowledge which molecular properties and pharmacokinetical parameters should be optimized (Pardridge, 2003; Jeffrey and Summerfield, 2007). The BBB represents an efficient structural and functional barrier for the delivery of therapeutic agents into the CNS. Due to its unique properties, passage across the BBB often becomes the main limiting factor for the delivery of potential CNS drugs into the brain parenchyma. In fact, it has been estimated that more than 98% of small-molecular weight drugs developed for the CNS diseases do not readily cross the BBB (Pardridge, 2005a).

The BBB endothelial cells differ from endothelial cells in the rest of the body by the presence of tight junctions, lack of fenestrae and the low frequency of pinocytic vesicles (Rubin and Staddon, 1999). There are also numerous enzymes and efflux-proteins present at the cerebral endothelial cells (Cordon-Cardo et al., 1989; Anderson, 1996). Due to these distinctive features of the

BBB, it exhibits an efficient barrier for the penetration of drugs into CNS. However, as each neuron in the human brain is perfused by its own blood vessel, a solute that is able to cross the BBB is distributed rapidly into the whole brain tissue (Pardridge, 2002a). Therefore, it is essential to develop new strategies to circumvent the BBB. The primary route of drug uptake into cells has been considered to be passive diffusion through the lipid bilayer of the cellular membrane. Recently it was reported that there is significant evidence supporting the view that drug uptake is, in fact, mainly transporter mediated (Dobson and Kell, 2008). Specific endogenous influx transporters have been identified at the brain capillary endothelium, the cells forming the BBB. These include large neutral amino acid transporter (LAT1) and glucose transporter type 1 (GluT1) (Pardridge and Oldendorf, 1977). The new awareness of these endogenous BBB transporters can be used in the rational modification of drug molecules for carrier-mediated transport (Rautio et al., 2008). In the view of the successful introduction of L-dopa over three decades ago, it is surprising that utilization of BBB transporters has not been more widely exploited in overcoming the barriers to CNS penetration. However, the complexity of this approach is often underappreciated, since it requires a sophisticated knowledge of CNS anatomy and physiology, as well as complex chemistry if it is to be successful.

The main objective of the present study was to prove that LAT1 and GluT1, which are both present at the BBB, can be utilized for brain drug delivery by conjugating drugs with endogenous substrates or substrate analogs of the transporters. It was hypothesized that by exploiting transporters the BBB permeation properties of drugs could be improved without increasing the non-specific tissue binding into the brain. However, good *in vitro* or *in situ* BBB permeability of drug molecules does not itself guarantee adequate brain uptake *in vivo*. Therefore, the second objective of the study was to prove that the nutrient promoieties are able to affect the systemic

pharmacokinetics of the drug molecules and increase the brain uptake *in vivo*.

2 Review of the literature

2.1 GENERAL BACKGROUND OF CENTRAL NERVOUS SYSTEM DRUG DELIVERY

The CNS includes the brain and the spinal cord. There are three barriers that limit drug transport into the brain (de Boer and Gaillard, 2007). These are the BBB, the blood-cerebrospinal fluid barrier (BCSFB), and the ependyma. The most important influx barrier preventing solutes from entering the brain is the BBB. The BBB is localized in the capillaries of the brain. The human BBB has an estimated surface area of approximately 20 m^2 (Keep and Jones, 1990). BCSFB attributable to the choroid plexus epithelium in the ventricles and the surface area is approximately the same as the surface area of the BBB. However, a large surface area of the BCSFB faces the cerebral spinal fluid (CSF), not blood, and therefore, the BCSFB is not as important influx barrier for CNS drugs as the BBB. Ependyma is an epithelial layer of cells covering the brain tissue in the ventricles and it limits the transport of compounds from CSF to the brain tissue (Bruni, 1998).

The delivery of drugs into the CNS is difficult to achieve although brain is highly perfused by capillaries and every cubic centimeter of cortex contains 1 km of blood vessels (de Boer and Gaillard, 2007). The entry of molecules from blood to brain is efficiently governed by the BBB and only small lipophilic molecules are able to cross the BBB by passive diffusion. It has been estimated that more than 98% of all potential new CNS drugs do not readily cross the BBB in sufficient amounts to have the desired pharmacological effect (Pardridge, 2003) (Fig. 2.1). In addition to brain tissue being a hard to reach for drugs, it has

been far from obvious what parameters should be used to determine adequate brain uptake of drugs (Jeffrey and Summerfield, 2007; Hammarlund-Udenaes et al., 2008). The brain to plasma concentration ratio (K_P) has been the most widely used parameter to evaluate and optimize the brain uptake during the drug discovery process (Liu et al., 2008). The current optimization paradigm, where better brain penetration has been viewed as increasing K_P of the drugs, may be incorrect, since higher K_P ratios are often a consequence of increased non-specific binding to brain tissue. In recent publications, the unbound brain to plasma concentration ratio ($K_{p,free}$ or $K_{p,uu}$) has been suggested to be a more important parameter in brain uptake optimization than K_P (Hammarlund-Udenaes et al., 2008; Liu et al., 2008). In addition, as drug efficacy is ultimately characterized by the relationship between the effect and drug concentration in the target tissue, the benefits of brain to blood concentration ratio optimization may be marginal in CNS drug discovery (de Lange, 2005; Jeffrey and Summerfield, 2007).

The global CNS drug market is highly under-penetrated. It has been estimated that one out of every three individuals will suffer a CNS condition during their lifetime, which means that the market of CNS drugs should be the largest sector in the pharmaceutical industry (Regier et al., 1988). However, the global CNS pharmaceutical market would have to grow more than 500% simply to match the share of the market occupied by cardiovascular drugs (Pardridge, 2002b).

The most challenging CNS diseases for drug treatment are neurodegenerative diseases, characterized by age-related gradual decline in neurological function (Pardridge, 2002b). These are for example, Alzheimer's disease, Parkinson's disease and Huntington's disease. Non-neurodegenerative diseases are often easier to treat and respond to small molecular weight drug treatments (Pardridge, 2005a). In addition, these CNS diseases are often not age-related. Examples of CNS diseases that respond to small molecular weight drugs are affective disorders, chronic pain, migraine and epilepsy.

The underlying reason for under-penetrated CNS drug market is the lack of efficient delivery strategies that enable drugs to circumvent the BBB. New knowledge on the structure and function of the BBB and the parameters describing brain uptake have provided an opportunity for the development of new strategies to overcome the CNS delivery problem. However, these new CNS targeting strategies have to be integrated into the drug design process at a very early stage. One promising strategy, which is also utilized in the present thesis are prodrugs. Prodrugs are compounds that undergo a chemical or enzymatic biotransformation prior to their therapeutic activity (Rautio et al., 2008). Release of the active drug is controlled and can occur before, during or after absorption, or at the specific site of action within the body, depending upon the purpose for which the prodrug is designed (Stella et al., 1985).

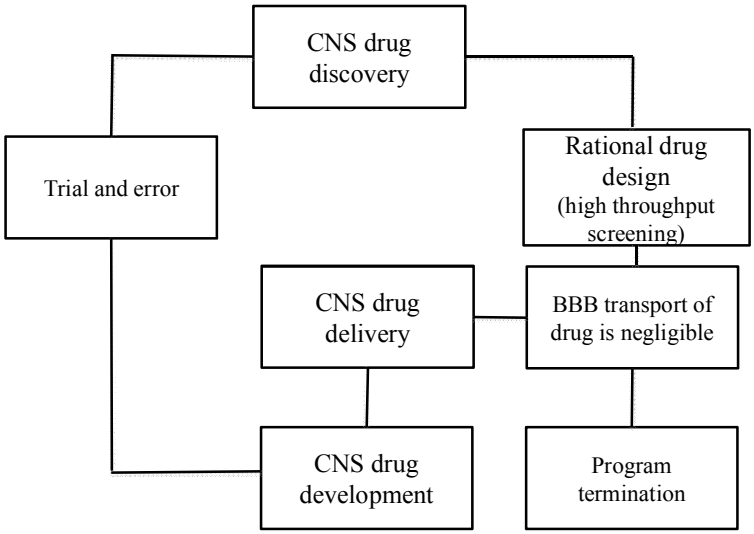


Figure 2.1. A schematic illustration of CNS drug discovery process. Virtually all drugs developed from receptor-based high throughput–screening programs for CNS drug discovery are either water soluble with a high degree of hydrogen bonding or

have a molecular weight greater than 400–500 Da. Without a rational CNS targeting strategy implemented in the drug discovery process, the drug design program is often terminated, because of poor brain uptake. Modified from (Pardridge, 2001b).

2.2 STRUCTURE AND FUNCTION OF THE BLOOD-BRAIN BARRIER

The BBB is essential for all animals with a complex CNS since it prevents the free movement of materials between the blood and the brain (Fig. 2.2). The BBB regulates the movement of solutes from blood to brain and it buffers the brain interstitial fluid from fluctuations in composition that occur in plasma (Braun et al., 1980; Begley, 2004b). In addition, the low permeability of the BBB to most neurotransmitters allows separation of the CNS and peripheral nervous system transmitter pools. Since it controls the movement of molecules from blood to brain, the BBB allows the precise regulation of solute concentrations in the interstitial fluid, which is essential for the propriate function of the CNS. The BBB is present in all brain regions, except for the circumventricular organs, where blood vessels have fenestrations that permit diffusion of solutes from blood to brain across the vessel wall (Ballabh et al., 2004). The unprotected areas of the brain regulate autonomic nervous system and endocrine glands of the body. The diffusion barrier of the BBB is due to endothelial cells with their continuous tight junctions (Pardridge, 2007b). In addition, the cells surrounding brain capillaries, such as astrocytes, pericytes, perivascular microglia and neurons contribute to the formation and maintenance of a functional BBB in the CNS (Goldstein, 1988; Dohgu et al., 2005; Nakagawa et al., 2007). Since the BBB blocks the passive diffusion of hydrophilic molecules, the efflux of hydrophilic metabolites formed in the CNS and the influx of hydrophilic nutrients are restricted. Therefore, there are several endogenous transporter mechanisms present at the BBB, which can facilitate the movement of both hydrophilic and large molecules across

the BBB (Pardridge, 1999a; Tsuji and Tamai, 1999; de Boer and Gaillard, 2007; Pardridge, 2007c). There is also high enzymatic activity in the cells forming the BBB, which can efficiently metabolize bioactive molecules before they cross the BBB and gain access to the brain parenchyma (Pardridge, 2005b).

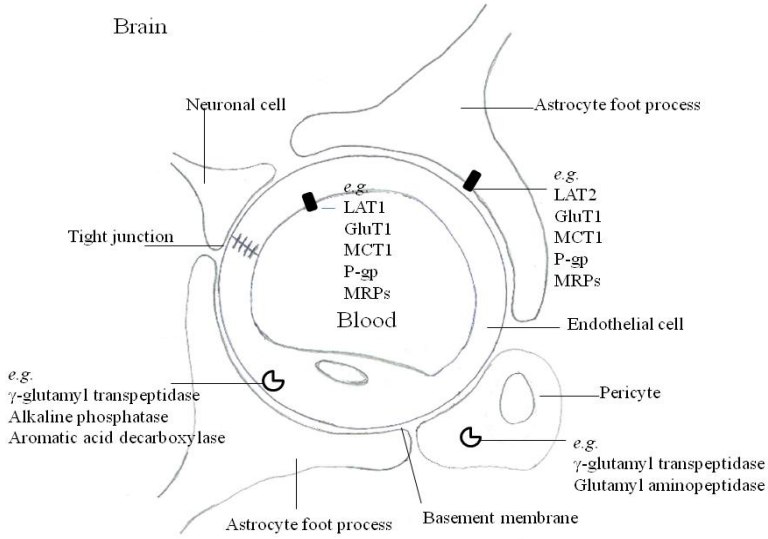


Figure 2.2. The structure of the blood-brain barrier.

Endothelial cells

The BBB is formed by a continuous layer of endothelial cells which form a very thin but very effective barrier between blood and brain parenchyma. Since the distance between luminal and abluminal membranes of endothelial cells is only 200 nm, this allows substances to cross the endothelial cells and enter the brain parenchyma within a short time (Stewart et al., 1985; Pardridge, 2005a). The brain capillary endothelial cells differ from the endothelial cells in the rest of the body, by having very little of pinocytotic and transsytotic activity and by their large number of mitochondria, suggesting their high energy metabolism (Oldendorf et al., 1977; Engelhardt, 2003). Transcytosis of molecules across the BBB is an adenosine

triphosphate (ATP) -dependent transport process and this enhanced energy potential may be related to energy-dependent transport across the BBB. The basement membrane of the BBB endothelial cells is common with that of the perivascular astrocytic endfeet and that of the pericytes, which are completely surrounded by a basement membrane, making the endothelial cells tightly integrated to the brain parenchyma (Allt and Lawrenson, 2001; Ballabh et al., 2004; Wolburg et al., 2009). The luminal surface of cerebral endothelial cells carries a negative charge due to negatively charged proteoglycans, glycosaminoglycans, glycoproteins and glycolipids on the surface of the cells (Fatehi et al., 1987; Brightman and Kaya, 2000; Begley and Brightman, 2003). This 25 nm thick glycocalyx covering the endothelial cells is a major resistance barrier to the passage of small solutes (Brightman and Kaya, 2000). In addition, cerebral endothelial cells express a wide spectrum of enzymes such as γ-glutamyl transpeptidase, alkaline phosphatase, butyrylcholine esterase, and aromatic acid decarboxylase, thus creating an enzymatic barrier between blood and brain (Betz et al., 1980; Anderson, 1996; Pardridge, 2005b). Furthermore, BBB endothelial cells express several transporter proteins, including P-glycoprotein (P-gp) (Cordon-Cardo et al., 1989; Thiebaut et al., 1989), multidrug resistance-associated proteins (MRPs) (Borst et al., 2000), GluT1 (Farrell and Pardridge, 1991), LAT1 (Boado et al., 1999; Duelli et al., 2000), the monocarboxylic acid transporter 1 (MCT1) (Gerhart et al., 1997), cationic amino acid transporter (y^+) (O'Kane et al., 2006) and the adenosine transporter (CNT2) (Li et al., 2001).

Tight junctions

The most important factors responsible for the restriction of the paracellular diffusion across the BBB are the junctional complexes which arwe present between the endothelial cells (McCaffrey et al., 2007). Tight junctions encircle the endothelial cells and the membranes of adjacent endothelial cells are completely fused. Therefore, the tight junctions between

adjacent endothelial cells are 50–100 times tighter than those encountered in peripheral endothelium (Abbott, 2002). In addition to sealing the paracellular route across the BBB, tight junctions are responsible for the polarization of the endothelial cells, which is manifested by a non-uniform distribution of transporters between the luminal and abluminal membranes (McCaffrey et al., 2007). Tight junctions are large, multiprotein complexes and the structure of the tight junction in the BBB has been found to be the most complex of all such entities in the entire vasculature of the body (Forster, 2008). The molecular components of tight junctions can be divided into different classes based on their structures and functions, including integral membrane proteins and cytoplasmic accessory proteins (Ballabh et al., 2004; Wolburg et al., 2009). Cytoplasmic proteins link membrane proteins to actin, which is the primary cytoskeleton protein involved in the maintenance of structural and functional integrity of the endothelium.

Astrocytes

Astrocytes encircle 90-99% of the capillaries formed by the endothelial cells (Pardridge, 2005a). In addition, astrocytes are attached to a basement membrane shared with the endothelial cells (Ballabh et al., 2004). However, the endfoot processes are not sealed to each other and the small gaps between the astrocytes allow passage of large and hydrophilic molecules. Although astrocytes do not take part in the formation of the physical barrier of the BBB, they are important in the development and maintenance of the BBB (Wolburg et al., 2009). Astrocytes induce and modulate the development of the BBB and its unique endothelial cell phenotype. *In vitro* studies have demonstrated, that astrocyte - endothelial cell interactions enhance endothelial cell tight junctions and reduce gap junctional area (Tao-Cheng et al., 1987; Tao-Cheng and Brightman, 1988; Wolburg et al., 1994). It has been reported that astrocytes are important for the expression of several transporter proteins in the brain endothelial cells, such as LAT1,

GluT1 and P-gp (El Hafny et al., 1997; Hayashi et al., 1997; Omidi et al., 2008). Moreover, the expression of several enzymes at the BBB is induced by astrocytes (Beck et al., 1986; Tontsch and Bauer, 1991). Therefore, astrocytes can play a major role in the BBB metabolism.

Pericytes

Pericytes are undifferentiated, contractile connective tissue cells that develop around capillary walls and share the basal membrane with brain capillary endothelial cells (Allt and Lawrenson, 2001; Persidsky et al., 2006). In addition, gap junction communication between pericyte and endothelial cells has been demonstrated *in vitro* (Larson et al., 1987; Lai and Kuo, 2005). Furthermore, pericytes are essential in structural differentiation of the brain endothelial cells, and formation of endothelial tight junctions (Nakagawa et al., 2007). Cerebral pericytes express several enzymes, such as γ-glutamyl transpeptidase and glutamyl aminopeptidase, therefore constituting a major component of the metabolic BBB (Frey et al., 1991; Song et al., 1993). In addition, it has been suggested that cerebral pericytes have phagocytotic potency (Jordan and Thomas, 1988).

2.3 MECHANISMS AFFECTING THE BRAIN UPTAKE OF DRUGS

The BBB represents an efficient barrier for the brain uptake of neuropharmaceuticals (Fig. 2.3). In addition to the optimal physicochemical properties, adequate brain uptake also requires that the drugs cannot be substrates of efflux proteins that are expressed on the luminal membrane of the endothelial cells.

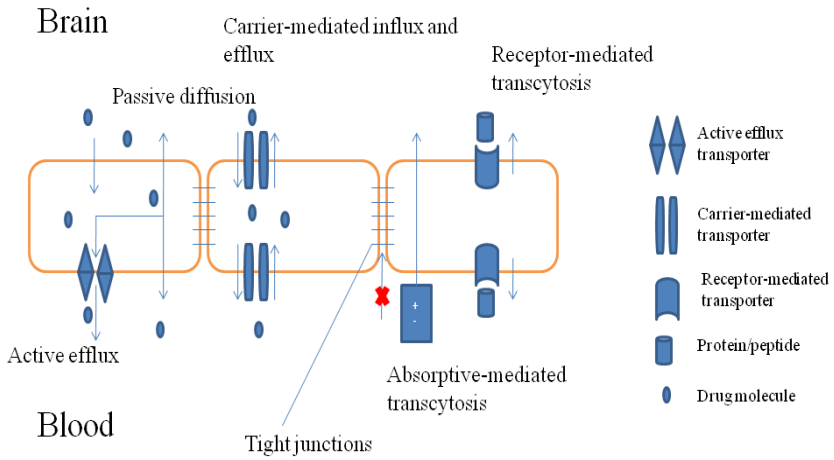


Figure 2.3. Mechanisms of brain uptake.

2.3.1 Passive diffusion

Passive diffusion across the BBB is believed to be the most common mechanism of CNS drug brain uptake. Although the importance of transporter mediated brain uptake may be underestimated. Passive diffusion involves an energy independent movement of drug molecules along a concentration gradient, and the rate of diffusion is directly proportional to the concentration gradient of the solutes across the membrane. Passive diffusion can occur either between the cells (paracellular) or through the cells (transcellular), depending on the physicochemical properties of the solutes. Since tight junctions block the paracellular route across the BBB, only solutes which are able to penetrate through the endothelial cell membrane are able to cross the BBB via passive diffusion. Therefore, only a few drug molecules are able to efficiently cross the BBB by passive diffusion. It has been suggested that the BBB permeation by passive diffusion is restricted to those molecules

which possess the criteria listed in Table 2.1. Examination of a comprehensive medicinal chemistry database revealed that the average molecular weight of the CNS active drug is 357 Da (Ghose et al., 1999). In a more recent study it was reported that the mean molecular weight of 74 CNS-drugs launched between years 1983-2002 was 310 Da (Leeson and Davis, 2004). This supports the limit for molecular weight presented in Table 2.1.

Table 2.1. The criteria for passive permeation of drugs across the BBB.

Properties	Limit[a]	CNS drugs [b]		
		average	min	max
Molecular weight (Da)	<450	298	129	654
Hydrogen bond donors	<3	1.03	0	4
Hydrogen bond acceptors	<7	3.33	1	9
CLogP	<5	2.55	-2.38	5.79
Polar surface area (Å^2)	<90	45.98	3.24	115.54

[a] according to (Pajouhesh and Lenz, 2005)

[b] CNS drugs marketed in Finland according to Pharmaca Fennica 2010. The properties of the drugs were calculated with ChemBio3D Ultra 12.0.

The average properties of CNS-drugs marketed in Finland fit well in the limits presented in Table 2.1. There are few exceptions in every category of properties and out of 129 drugs 13 molecules do not fulfill all the properties in Table 2.1. The mechanism of brain uptake of these drugs may be other than passive diffusion. For example, L-dopa, which has PSA of 103.78

Å^2, utilizes LAT1 for brain uptake (Gomes and Soares-da-Silva, 1999b). Another Parkinson`s disease drug bromocriptine has molecular weight of 654.53 Da and PSA of 114.45 Å^2 and is still able to cross the BBB, although the mechanism of brain uptake is unknown. Three drugs with sulfonamide functional group, sulpiride, sulthiame and topiramate, have PSA over the limit presented in the Table 2.1 and the passive diffusion across the BBB may be limited. In fact, sulpiride has been reported to be substrate of organic cation transporter 2 (OCTN2) in Caco-2 cell line (Watanabe et al., 2002). OCTN2 has been reported to be present at the BBB, and may mediate the brain uptake of sulpiride, sulthiame and Topiramate (Friedrich et al., 2003; Inano et al., 2003).

Although small lipophilic molecules are able to cross the BBB by passive diffusion, those molecules may encounter other limitations. Lipophilic drugs are often highly bound to brain tissue (Summerfield et al., 2007). The non-specific binding of the drugs to the brain tissue enlarges the distribution volume of the drugs in the brain parenchyma, which sustains the blood to brain concentration gradient and enhances brain uptake (Liu et al., 2005; Summerfield et al., 2007). However, as only the free fraction of the drug is effective, the high brain uptake due to the non-specific brain tissue binding is futile. In recent years, drug development has focused on optimizing the drug-target protein interactions, which has lead to the development of large molecules, which do not fulfill the requirements for passive BBB permeation (Pardridge, 2003). Furthermore, only a few of the drugs that are active in the CNS have been developed for illnesses other than affective disorders, because only a few brain diseases consistently respond to lipid-soluble small molecules (Ajay et al., 1999; Ghose et al., 1999; Lipinski, 2000). Therefore, it can be postulated that brain uptake mechanisms other than passive diffusion may become more significant in CNS drug development in the future.

2.3.2 Carrier-mediated transport

The many transporter processes present in the cerebral endothelial cells enable the movement of hydrophilic and large molecules across the BBB (Tsuji and Tamai, 1999; Pardridge, 2007c). Carrier-mediated transporter proteins move small hydrophilic molecules such as amino acids and glucose. Some transporters are unidirectional in their transport of solutes across the cell membrane and move solutes either from brain to blood or from blood to brain. Some transporters are bidirectional, and therefore, transport of some solutes can be facilitated in either direction depending on whether the concentration gradient across the BBB is directed into or out of the CNS (Meier et al., 2002; Begley, 2004a; Tsuji, 2005).

Carrier-mediated transporters can be divided into active transporters and equilibrative transporters. Active transporters are either primary or secondary active. Primary active transporters have intracellular ATP binding sites whereas secondary active transporters require the presence of an ion gradient to facilitate the transport of molecules (O'Kane et al., 2004; Dallas et al., 2006). Equilibrative transporters do not require energy. However, unlike active transporters the equilibrative transporters are not able to move solutes against a concentration gradient. The function of carrier-mediated transporters is temperature dependent and their activity can be influenced with competitive or non-competitive inhibitors (Blodgett and Carruthers, 2005). Moreover, carrier-mediated transporters can be saturated and their uptake follows Michaelis-Menten kinetics. The BBB transporters are expressed on the luminar and/or abluminal membranes of the endothelial cells depending on the transporter. It has been suggested that carrier-mediated transporters are able to move molecules which have a molecular mass below 600 Da (Pardridge, 2001a). However, the actual limit of the molecular mass may vary depending on the transporter. Carrier-mediated transporters facilitate the uptake of various essential nutrients into the CNS, including amino acids, glucose, vitamins and nucleosides

(Pardridge and Oldendorf, 1977; Boado et al., 1999; Chishty et al., 2004; Cornford and Hyman, 2005; Park and Sinko, 2005), since their brain supply would be restricted without the presence of the transporters in the endothelial cells (Table 2.2). As many drug molecules have similar structural properties to endogenous substrates, it is clear that some membrane transporters can participate in drug transport (Tamai and Tsuji, 2000). Two carrier-mediated transporters, GluT1 and LAT1, will be discussed in more detail, since these transporters are considered as the most promising transporters to be utilized for brain drug delivery with prodrug technology (Walker, 1994; Halmos et al., 1996; Bonina et al., 1999; Bonina et al., 2003; Fernandez et al., 2003).

LAT1

LAT1 has an important role in the maintenance of the normal function of the mammalian brain, because the rates of amino acid incorporation into brain proteins by means of cerebral protein synthesis are about the same as the rates of amino acid influx across the BBB (Pardridge, 1998). In addition, the surface area of the brain cell membranes is significantly greater than the surface area of the BBB (Lund-Andersen, 1979). Therefore, the LAT1-mediated amino acid transport across the BBB is the rate-limiting step in amino acid movement from blood to brain intracellular spaces (Boado et al., 1999). LAT1 transfers one amino acid out of the cell while another amino acid is transported into the cell (Verrey, 2003). The driving force of LAT1 is provided by a Na^+-dependent amino acid transporter that carries an amino acid that is a common substrate for both systems. However, the dynamics of the whole system are not yet fully understood. LAT1 is only able to modify the relative concentrations of different substrate amino acids, and cannot induce a change in the overall intracellular amino acid concentration. Therefore, the net direction of the transport of amino acids is believed to depend on the unidirectional Na^+-dependent transporters that are co-expressed in the cells. Since

LAT1 is expressed in parallel to the unidirectional transporters at the BBB, LAT1 can participate in the flux of amino acids from the blood to the brain or, under certain circumstances, from the brain to the blood (Sanchez del Pino et al., 1995; Ennis et al., 1998). LAT1 is expressed on the luminal and abluminal membranes of brain capillary endothelial cells (Verrey, 2003). In addition, LAT1 is also expressed in testis, placenta and tumours (Kanai et al., 1998; Yanagida et al., 2001). This suggests that LAT1 is involved mainly in the transport of amino acids into growing cells and across some endothelial and epithelial barriers. The amount of LAT1 mRNA in bovine brain capillary endothelial cells determined with Northern blotting experiments is approximately 100-fold greater compared to other tissues, such as lung, spleen, testis, and heart (Boado et al., 1999). In addition, the level of LAT1 mRNA was higher relative to GluT1 mRNA at the BBB. However, the higher level of mRNA may not correlate with a higher level of LAT1 compared to GluT1, since the maximum transport velocity (V_{max}) of GluT1 is significantly higher than the V_{max} of LAT1 (Pardridge, 2001b). However, the abundant LAT1 mRNA at the BBB may mean that this transcript has a high turnover rate (Boado et al., 1999). It has been proposed that one regulation mechanism of LAT1 gene expression at the BBB may be posttranscriptional and that the regulation of BBB LAT1 gene expression may play an important role in the adaptive response of the brain to an abnormal plasma amino acid supply.

The affinity of large neutral amino acids for LAT1 at the BBB is much higher than the affinity of amino acids for the other L-system transporters in peripheral tissues (Boado et al., 1999). In humans the Michaelis constant (K_m) for LAT1 at the BBB is 10-100 μM, whereas the K_m for peripheral amino acid transporters is 1-10 mM. In addition, the Km of LAT1 at the BBB is similar to the plasma concentration of circulating large amino acids, which means that this transporter is saturated under normal conditions (Pardridge, 1986). LAT1 preferentially transports large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine and histidine (Boado et al., 1999; Duelli et al., 2000). An

analysis of the structures of the LAT1 substrates revealed that LAT1 substrates need to possess an amino group, carboxyl group and hydrophobic side chain in order to be recognized by LAT1 (Fig. 2.5) (Uchino et al., 2002). Results from affinity tests suggest that by removing either the amino group or the carboxyl group of leucine and phenylalanine, the affinity for LAT1 is lost. However, by conjugating LAT1 substrate from the side chain to a drug molecule, the affinity can be sustained. This information can be utilized for rational design of drugs and prodrugs that are then able to penetrate the BBB via the LAT1. In addition, since LAT1 expression is up-regulated in rapidly dividing tumor cells in order to supply these cells with essential amino acids to meet their need for continuous growth and proliferation, it may be possible to impair the growth of tumors by inhibiting LAT1 activity (Langen et al., 2001).

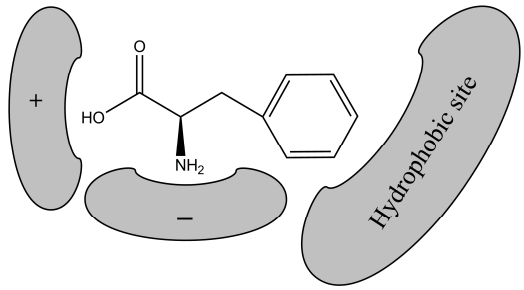


Figure 2.5. A simplified illustration of LAT1 binding site (Uchino et al., 2002; Smith, 2005). The illustration is heavily simplified. However, because of lack of crystal structure for LAT1 the simplified model serves as a good template for drug and prodrug design when the aim is to utilize LAT1.

GluT1

GluT1 transports D-glucose, which is the main energy source of brain, across the BBB and then further into the neuronal cells (Mueckler, 1994). It has been estimated that the glucose consumption of the human brain is 30% of the entire body glucose consumption, and the brain endothelium transports

about ten times its weight in glucose per minute (Dick et al., 1984; LaManna and Harik, 1985). GluT1 mediates energy independent transport of glucose, which leads to glucose equilibration, but not glucose accumulation, by cells. Moreover, GluT1 is a bi-directional transporter, and the presence of intracellular and/or extracellular glucose alters the kinetics of transport both in and out of the cell (de Graaf et al., 2001; Qutub and Hunt, 2005; Simpson et al., 2007). The density of glucose transporters in the BBB endothelium is three to four times higher in the abluminal than in the luminal membrane (Farrell and Pardridge, 1991). There are two types of glucose transporters, namely sodium -dependent and -independent transporters (Nishizaki et al., 1995; Wright et al., 1997; de Graaf et al., 2001). Sodium-independent glucose transporters are thought to be functional in the brain, although some studies claim that sodium-dependent glucose transporters may also be present in the brain (Nishizaki et al., 1995). Two different molecular weight forms (45 and 55 kDa) of GluT1, due to different extents of glycosylation, have been detected in mammalian brain (Birnbaum et al., 1986). However, their protein structure or kinetic characteristics are similar. The V_{max} of GluT1 is 1420 nmol/min × g tissue and the transporter capacity is estimated to be 15–3000 -fold higher than that for other transporters present at the BBB, such as MCT1 and LAT1 (Pardridge, 1983). Due to the high capacity of GluT1 at the BBB, it is expected to be applicable for the brain delivery of drugs (Pardridge, 1983). Furthermore, there are current data which can be used to create a model for the exofacial configuration of GluT1 in which transmembrane segments form an inner helical bundle that comprises a water-accessible cavity within the membrane (Fig. 2.6) (Mueckler and Makepeace, 2008). This knowledge of the glucose binding site allows the rational design of glucose analogs as well as prodrugs, which can utilize GluT1 for enhanced BBB permeation. In addition, as GluT1 ensures the insatiable glucose consumption of some cancer cell types, it could be useful to inhibit the function of GluT1 in these cells (Amann et al., 2009; Ganapathy et al., 2009).

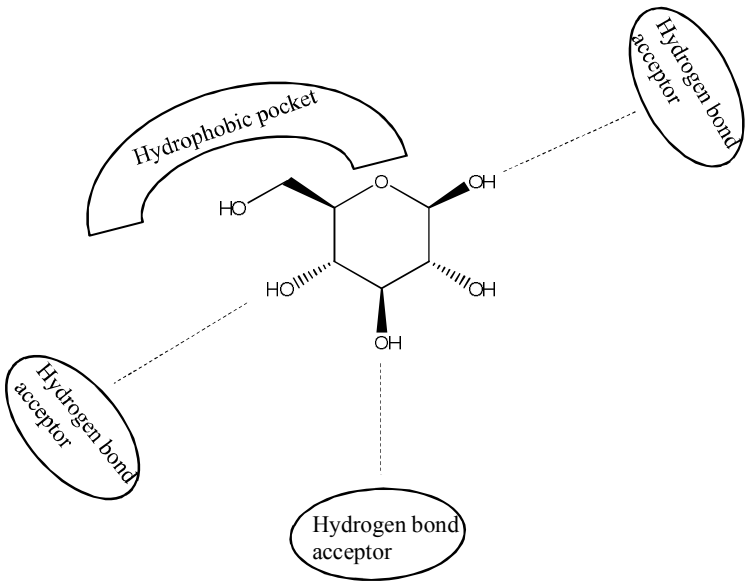


Figure 2.6. A proposed and simplified model of the exofacial glucose-binding site of GluT1. Dotted lines represent the hydrogen bonds between the transporter and glucose (Mueckler and Makepeace, 2008). This simplified model of the binding site can be used for glucose prodrug, because it shows which hydroxyl groups are important for the substrate binding.

2.3.3 Receptor-mediated transport

Brain uptake of large molecules such as peptides and proteins is limited due to the BBB. The endocytotic activity of BBB endothelial cells is lower than in the peripheral endothelial cells (Rubin and Staddon, 1999). However, the brain uptake of some large molecular weight molecules is necessary to ensure the normal function of the brain. Therefore, some peptides and proteins gain their access into the CNS via receptor-mediated transport or nonspecific absorbtive mediated transcytosis (Fig. 2.3) (Begley, 2004b). Brain influx of nutrients, such as iron, insulin and leptin is mediated by transferrin receptor, insulin receptor, and leptin receptor, respectively (Jefferies et al., 1984;

Duffy and Pardridge, 1987; Golden et al., 1997). Transcytosis occurs when circulating ligand interacts with a specific receptor at the luminal membrane of the endothelial cell (Brown and Greene, 1991). In receptor-mediated endocytosis, the uptake of particles or ligands is saturable because it is dependent upon the extracellular availability of receptors (Mellman, 1996; de Boer, 2003). This receptor-ligand binding then induces an endocytic event in the luminal membrane that probably involves aggregation of receptor-ligand complexes and triggers the internalization of an endocytotic vesicle containing the receptors and the attached protein molecules. This process requires energy and is also temperature sensitive. In addition, the internalization process is time dependent. These internalized vesicles can then enter a pathway, which carries them across the endothelial cell during which the peptide/protein is dissociated from the receptor and exocytosed at the luminal membrane of the endothelial cell, resulting in transport across the BBB (Begley, 2004b). In this way, molecules can cross the endothelium and enter the brain without disruption of the barrier properties. While receptor-mediated transport systems are selective pathways for trans-BBB transport in that they require the initial binding of a ligand to some moiety on or in the plasma membrane of the endothelial cells that make up the BBB, nonspecific absorbtive mediated transcytosis relies on nonspecific charge-based interactions (Bickel et al., 2001). Nonspecific absorbtive mediated transcytosis can be initiated by polycationic molecules binding to negative charges on the plasma membrane (Pardridge et al., 1990). In contrast to carriers, receptors are able to internalize relatively large compounds and systems and are therefore more suited for targeted drug delivery of peptides, proteins, and even nanoparticles to the brain (Munn, 2001).

Receptor-associated protein (RAP) is found mainly in the endoplasmic reticulum (Pan et al., 2004). RAP plays a key role in the proper folding and trafficking of members of the low-density lipoprotein receptor family within the secretory pathway, including low-density lipoprotein receptor-related

protein 1 (LRP1) and low-density lipoprotein receptor-related protein 2 (LRP2) (Bu et al., 1995). There are two main consequences to the existence of RAP transport across the BBB. First, the transport system could be enhanced to facilitate the transport of ligands such as RAP and its homologous proteins. An alternative approach is the use of a transport system with a ligand as a carrier protein. Therefore, it may be possible to attach the target drug to RAP to provide efficient transport into the brain. It was reported by (Pan et al., 2004) that RAP crosses the BBB by an efficient, saturable transport system probably mediated by megalin. In this process, RAP enters the brain parenchyma intact. Identification of a specific transport system for RAP at the BBB has highlighted the potential for RAP-mediated delivery of therapeutic peptides and proteins from blood to brain.

The principle of using BBB transport mechanisms can be also applied to large peptides and proteins that may use either receptor-mediated transcytosis or non-specific absorbtive mediated transcytosis (Begley, 2004b). Receptor-mediated transytosis -mediated drug delivery also takes advantage of the endogenous BBB-transport systems, and aims to improve brain uptake by coupling non-transportable therapeutic molecules to a drug-transport vector (Pardridge, 1999b; Bickel et al., 2001). A drug-transport vector may include endogenous peptides, such as insulin or transferrin, a modified protein, or it may include anti-receptor specific monoclonal antibodies (MAb) that undergo transcytosis through the BBB via the endogenous receptor system within the brain capillary endothelium. Conjugation of a drug to a transport vector can be facilitated either by chemical linkers, avidin-biotin technology, polyethylene glycol linkers, or liposomes. The MAb binds an exofacial epitope on the receptor that is spatially removed from the binding site of the endogenous ligand and this receptor binding allows the MAb to piggy back across the BBB via the endogenous receptor-mediated transytosis system within the BBB. The receptor-specific MAb may act as a molecular Trojan horse and ferry any attached drug or non-viral plasmid DNA

across the BBB. By employing a MAb to the receptor as the vector rather than the substrate protein itself, the strategy avoids the endogenous substrate in blood competing for receptors at the BBB (Bickel et al., 1994). The capacity of this system for delivery is generally quite low, as the use of a vector in this manner results in only one molecule of peptide/protein being delivered per transferrin receptor antibody (Begley, 2004b).

2.3.4 Active efflux transport

Active efflux transporters have a major impact on the drug systemic pharmacokinetics in the body (Fromm, 2000; Loscher and Potschka, 2005). The transcellular brain uptake of some small lipophilic solutes is not as high as would be indicated by their lipophilicity (Levin, 1980; Kusuhara and Sugiyama, 2001). The lower brain uptake of lipophilic solutes is often due to active efflux proteins, such as P-gp, that remove solutes from endothelial cells (Loscher and Potschka, 2005). The impact of efflux proteins on the brain uptake of CNS drugs is significant, because the efflux transporters have a broad range of substrates, and strong substrates of BBB efflux transporters do not pass the BBB to a functionally relevant extent, which restricts their therapeutic effects to the periphery. Although, active efflux transporters are very important factor in pharmacokinetics they are not discussed in more detail, because the scope of the present thesis is on the utilization of the influx transporters.

2.4 PARAMETERS AND METHODS USED TO STUDY AND ESTIMATE BRAIN PERMEATION OF DRUGS

2.4.1 Parameters describing drug uptake across the blood-brain barrier in animal models

Brain penetration kinetics can be described by the extent and time to reach brain equilibrium (Liu and Chen, 2005; Hammarlund-Udenaes et al., 2008). The lack of success in brain

drug delivery to date might be due to a lack of any consensus about regarding which processes and properties are most relevant to successful brain drug delivery. A combination of measurements has been proposed, as a single rapid method cannot map all the important factors. In the past, the optimization of K_P has been used as the parameter that describes best the extent of brain drug delivery in animal studies (Liu et al., 2008). However, several studies have shown that the optimization of K_P leads to non-specific brain tissue binding and the usefulness of this parameter has been criticized (Pardridge, 2004; Jeffrey and Summerfield, 2007; Summerfield et al., 2007). As it is generally accepted that it is the unbound drug that exerts the pharmacological effects, the extent should be defined as $K_{p,free}$ at steady state (Hammarlund-Udenaes et al., 1997; Syvanen et al., 2006; Hammarlund-Udenaes et al., 2008; Liu et al., 2008). In addition, the distribution of the free drug inside the brain compartments is crucial (Fig. 2.7). The comparative importance of unbound drug concentrations in different brain compartments, extracellular fluid (ECF) or intracellular fluid (ICF), depends on where the site of action is situated. If the drug in question is actively transported across the cell membrane, brain ICF concentrations could be expected to differ from brain ECF concentrations (Friden et al., 2007; Hammarlund-Udenaes et al., 2008). There is no direct method for measuring the concentration ratio between ICF and ECF ($K_{p,free,cell}$). However, recently an indirect technique was proposed, where by combining data from *in vitro* rat brain slice method with *in vivo* rat brain concentration the drug ICF concentration can be calculated (Friden et al., 2007). Furthermore, the pharmacological effects of CNS drugs are characterized by the relationship between efficacy and the drug concentrations at the active site (de Lange, 2005; Jeffrey and Summerfield, 2007). Therefore, the optimization of the partition ratio between plasma and brain is less important than the optimization of the absolute concentrations at the active site.

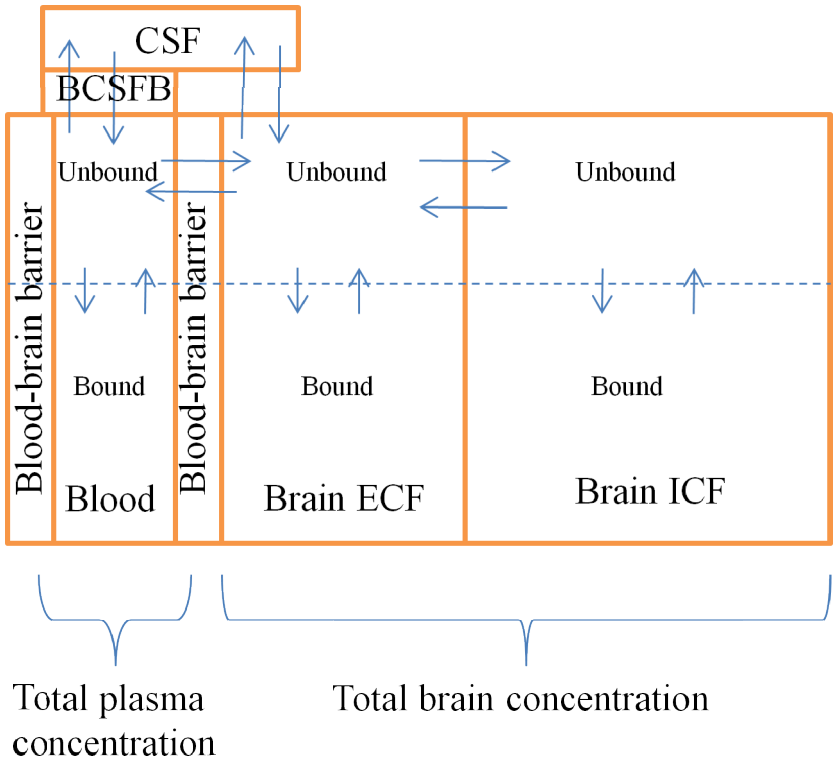


Figure 2.7. A schematic illustration of brain compartments (Hammarlund-Udenaes et al., 2008).

For some CNS drugs, the time to reach brain equilibrium is as important a parameter as the extent of brain permeation (Liu et al., 2005). Rapid brain penetration can be achieved by increasing BBB permeability and reducing brain tissue binding. Thereby the unbound drug concentration at the target site in brain tissue can reach equilibrium with the plasma unbound concentration rapidly after administration. The property of rapid brain penetration can be gauged by the time to reach brain equilibrium. Therefore, a short time to achieve brain equilibrium is a surrogate for a rapid achievement of active brain concentration. The rate of transport of a drug across the BBB is estimated as the PA product, or the influx clearance (K_{in}) which are clearance measurements and not rates per se (Equation 1) (Hammarlund-Udenaes et al., 2008).

$$K_{in} = \frac{q_{tot} - V_v \times C_{pf}}{T \times C_{pf}} \quad (1)$$

where q_{tot} is total brain concentration, V_v is the vascular volume of the brain, C_{pf} is the perfusion fluid concentration and T is the perfusion time. The transformation of K_{in} to the PA product is performed using the Crone-Renkin model (Equation 2) (Killian et al., 2007).

$$PA = -F \times ln[1 - (K_{in}/F)] \quad (2)$$

where F is the flow rate determined with lipophilic solute such as diazepam used as a marker for cerebral blood flow.

The time to achieve brain equilibrium can be quantitated with intrinsic brain equilibrium half-life ($t_{1/2eq,in}$), a parameter proposed by Equation 3 (Liu et al., 2005).

$$t_{1/2} = \frac{V_b \, ln2}{PA \times f_{u,brain}} \quad (3)$$

where V_b is the physiological volume of brain and fu,brain is the free fraction of the drug in brain tissue.
The distribution of drugs into other tissues than brain decreases the plasma concentration, and therefore might increase the K_p or $K_{p,free}$, although the brain uptake of the drug is actually decreased (Pardridge, 2003). Therefore, sometimes the optimization of $K_{p,free}$ can lead to decreased brain concentrations of drugs. The percent of injected dose of a drug that is delivered per gram brain (%ID/g) should be determined for CNS drugs, because %ID/g determines how large fraction of the drug dose is delivered into the brain instead of determining the distribution ratio between brain and blood. %ID/g is directly proportional to both the BBB PA product and the area under the plasma concentration curve (AUC) (Equation 4).

$$\%\frac{ID}{g} = PA \times AUC \qquad (4)$$

The fraction of unbound drug in the brain originates from the perception that drug distribution within the brain is largely dominated by non-specific binding, which can be determined by a brain homogenate binding technique (Kalvass and Maurer, 2002; Maurer et al., 2005; Friden et al., 2007). The parameter $f_{u,brain}$ is therefore the fraction of unbound drug in (undiluted) brain homogenate. The *in vivo* interpretation of actual unbound drug concentrations in ECF is difficult since the intact brain has distinct compartments i.e., the intra- and extracellular spaces. It cannot be directly assumed that the concentration of unbound drug in brain ECF equals that in brain ICF, as there are also transporters in the brain parenchymal membranes (Thurlow et al., 1996; Dallas et al., 2006). It is currently not possible to directly measure intracellular unbound drug concentrations, but indirect techniques are emerging from the combined use of rat brain slice uptake experiments and binding studies in homogenised brain (Friden et al., 2007). Due to the absence of plasma proteins in the brain ECF and the small fraction of membrane surface area that faces the ECF, drug binding in brain tissue can be considered as intracellular (Friden et al., 2007). By combining the brain homogenate binding techniques of intracellular binding with measures from brain slice uptake method, $K_{p,free,cell}$ can be calculated.

A previously described approach to account for the effect of tissue dilution on unbound fraction was used to calculate the brain unbound fraction (Equation 5) (Kalvass and Maurer, 2002).

$$f_{u,brain} = \frac{f_{u,homogenate}}{D-(D-1)f_{u,homogenate}} \qquad (5)$$

where D represents the -fold dilution of brain tissue, and $f_{u,homogenate}$ is the ratio of concentrations determined from the buffer and brain homogenate samples.

The unbound drug volume of distribution in the brain ($V_{u,brain}$) describes the relationship between the total drug concentration in the brain and the unbound drug concentration in brain ECF (Equation 6) (Hammarlund-Udenaes et al., 2008). $V_{u,brain}$ is measured in mL/g $_{brain}$:

$$V_{u,brain} = \frac{AUC_{brain}}{AUC_{u,brain\ ECF}} \qquad (6)$$

where AUC_{brain} (nmol/g $_{brain}$ × min) comprises the amount of unbound drug in the ECF and the amount of drug associated with the cells (Equation 7):

$$AUC_{brain} = V_{brainECF} \times AUC_{u,brainECF} + V_{cell} \times AUC_{cell} \qquad (7)$$

$V_{brainECF}$ and V_{cell} are the physiological fractional volumes of the brain ECF and brain cells, respectively (mL/g $_{brain}$), and AUC_{cell} is the amount of drug associated with the cells (nmol/mL$_{cell}$ × min). The distribution volume of unbound drug in the cell is described by $V_{u,cell}$ (mL$_{ICF}$/mL$_{cell}$) and the intracellular concentration of unbound drug is described by $AUC_{u,cell}$ (nmol/mL$_{ICF}$ × min) (Equation 8):

$$AUC_{cell} = V_{u,cell} \times AUC_{u,cell} \quad (8)$$

$V_{u,cell}$, describes the affinity of the drug for physical binding inside the cells (Friden et al., 2007), and it was estimated using the brain homogenate binding experiment and taking V_{cell} into account in the dilution factor (Equation 9):

$$V_{u,cell} = 1 + \frac{D}{V_{cell}} \left(\frac{1}{f_{u,hD}} - 1\right) \quad (9)$$

When combining $V_{u,cell}$ assayed from homogenate binding method and $V_{u,brain}$ acquired from *in vivo* microdialysis and *in vivo* whole tissue experiments or by using *in vitro* brain slice method, the ICF-to-ECF concentration ratio of unbound drug can be calculated as follows (Equation 10):

$$K_{p,free,cell} = \frac{C_{u,cell}}{C_{u,brainECF}} = \frac{V_{u,brain} - V_{brainECF}}{V_{cell} \times V_{u,cell}} \qquad (10)$$

2.4.2 *In vitro* methods

Generally *in vitro* methods offer some advantages over *in vivo* methods. These include a lower compound requirement, greater throughput, ability to assess mechanisms involved in the uptake process separately, the identification of early signs of cell toxicity, and finally lower cost (Lundquist and Renftel, 2002). In addition, the use of *in vitro* methods can decrease the use of laboratory animals, and more importantly reduce painful operations performed on animals, and therefore is ethical. *In vitro* methods for brain uptake studies can be divided into two groups: 1) *in vitro* methods that aim to accurately replicate the conditions involved in *in vivo* brain uptake, and 2) methods that are used to study separately the processes determining the brain uptake (Lasbennes and Gayet, 1984; Joo, 1993) (Gumbleton and Audus, 2001; Jeffrey and Summerfield, 2007).

The *in vitro* BBB models which aim to replicate *in vivo* conditions include isolated brain capillaries, primary and low passage brain capillary endothelial cell cultures, immortalized brain endothelial cells and immortalized artificial membranes (Lundquist and Renftel, 2002). An ideal *in vitro* model should have similar paracellular permeability and transporter characteristics as the *in vivo* human BBB and should be easily set up for routine drug screening (Liu et al., 2008). More research is needed to develop such an *in vitro* BBB model. To overcome the limitations of these cultured cells, extensive studies are being conducted to develop better *in vitro* BBB models using either primary animal brain endothelial cells or immortalized brain endothelial cells. Coculture with astrocytes has been used to mimic the *in vivo* environment (Li et al., 2010). To an attempt to better mimic *in vivo* situation, a dynamic coculture model in a hollow-fiber cartridge has been developed (Neuhaus et al., 2006). However, because of the complicated set up and low throughput of this dynamic model, it is difficult to use in drug

discovery in its current format. The major advantage with isolated brain capillaries is their close resemblance to the *in vivo* situation and they have indeed proved to be suitable for the detailed studying of the molecular interactions underlying several important endothelial functions. However, their major drawbacks from an industrial perspective are that they are not well suited for screening purposes. An immortalized cell line has the advantage of being less labour intensive (Cucullo et al., 2008). However, there are several disadvantages *e.g.* cells have been manipulated they form incomplete tight junctions and lack the necessary paracellular barrier properties in order to be considered as a BBB permeability screen (Rist et al., 1997). Transport studies reported with immortalized cell lines also have mainly been concerned with drug uptake in the cells rather than transendothelial drug transport. Madin-Darby canine kidney cells (MDCK) cells show low permeability to sucrose and this, in combination with the ease with which MDCK cells can be grown, have led to their proposed use as a BBB screen for passively transported CNS compounds (Lundquist and Renftel, 2002). There are also MDCK cells available, transfected with the human multi-drug resistance -1 gene leading to a polarized overexpression of P-gp, which has proved to be an important BBB efflux mechanism. However, the noncerebral cells are morphologically very different from brain endothelial cells and also differ with respect to transport properties, metabolism and growth. The morphological differences compared to cerebral endothelial cells are likely to be reflected in drug–cell membrane interactions resulting not only in different transport-mediated permeation but also transfer mediated by passive diffusion.

From the academic research point of view, it is not interesting to screen vast amounts of molecules. Instead, it is more important to study the processes that influence the brain uptake of drugs, and *in vitro* methods are suitable for this research. Three major factors have been suggested to determine the brain uptake of drugs (Jeffrey and Summerfield, 2007). These are passive membrane permeability, facilitated drug transport and the relative degree of tissue binding between blood and brain. These

processes act in concert and the determination of which of these processes dictate the extent of brain uptake is difficult *in vivo*. Therefore, *in vitro* methods are important for brain uptake research, because these three processes can be individually studied one at a time (Jeffrey and Summerfield, 2007). Several *in vitro* cell culture models have been developed for the means to study membrane permeation of potential CNS drug molecules (Di et al., 2009). In passive membrane permeation studies, it is important that the cell model does not express membrane transporters or enzymes involved on the drug metabolism (Jeffrey and Summerfield, 2007). In addition, tight junctions between the cells should be present, because they are a significant part of the *in vivo* BBB morphology.

If one wishes to determine the significance of transporter mediated efflux or influx, the cell model should express specific transporters. The rat brain endothelial cell line (RBE4) is probably the most extensively characterized cell line in which the endothelial cells have been transfected with a plasmid containing the E1A adenovirus gene. The RBE4 has been shown to functionally express a number of BBB transporters (Begley et al., 1996; Reichel et al., 2000). In addition to studying carrier-mediated uptake across continuous cell layers, the interactions between transporters and drug molecules and also carrier-mediated cellular uptake can be studied with cell suspensions such as freshly isolated human erythrocytes, which express GluT1 (Halmos et al., 1996; Fernandez et al., 2003). Furthermore, several transporters have been transfected and expressed in *Xenopus laevis* oocytes and used for transporter research (Uchino et al., 2002; Boado et al., 2005).

In vitro brain tissue binding technique in homogenized rat brain is based on an assumption that drug binding in brain tissue can be considered as being intracellular (Friden et al., 2007). Accordingly, the brain homogenate binding method measures intracellular binding and this allows the distribution volume of the free drug in the intracellular compartment to be estimated. When $V_{u,free,cell}$ is combined with distribution volume of free drug in the brain calculated from *in vivo* brain microdialysis and

whole brain tissue or from brain slice method, the $K_{p,free,cell}$ can be calculated.

2.4.3 *In situ* rat brain perfusion technique

The primary objective of *in situ* rat brain perfusion technique is to substitute for the blood circulation of the brain via direct infusion of perfusion medium into the major vessels leading to the brain, such as carotid arteries (Fig. 2.8) (Takasato et al., 1984). A known concentration of the solute of interest is infused into the brain at a desired infusion flow rate and time. After the perfusion, the amount of solute in the brain is determined and appropriate transport or permeability constants are calculated. A number of variations of the *in situ* brain perfusion technique have been published and some of the complexity of the method has been removed over the time (Foster and Roberts, 2000; Smith and Allen, 2003). The experimental conditions can be easily manipulated to study saturable processes such as carrier-mediated uptake, and moreover, experimental conditions that would be toxic in the *in vivo* situation can be used. The most important advantage over *in vivo* experiments is the simplicity of the pharmacokinetics permitting an accurate determination of the uptake mechanism possible (Smith and Allen, 2003; Summerfield et al., 2007). *In vivo* techniques take into account not only BBB penetration but also binding to plasma proteins, metabolism, and clearance. There is significant value in removing some of this complexity and assessing brain penetration at the level of the BBB *in situ*. The most important advantage over *in vitro* techniques is that the BBB is in its normal physiological state when the *in situ* technique is used.
In situ brain perfusion technique can be used to determine the mechanism of uptake of drugs. In addition, pharmacokinetic parameters such as V_{max} and K_m can be determined for carrier-mediated uptake. This is possible because the composition of the perfusion medium can be easily manipulated in terms of drug and/or transporter inhibitor concentration. Furthermore, the parameters describing the permeability of a drug across the

BBB, such as PA product or K_{in}, can be determined. In addition, by combining the PA product data acquired from *in situ* studies with *in vitro* unbound fraction in brain tissue, the time to reach brain equilibrium can be evaluated for CNS drugs (Liu et al., 2005). However, the estimation of the extent of the *in vivo* brain uptake is problematic, because systemic pharmacokinetics cannot be determined with *in situ* brain perfusion.

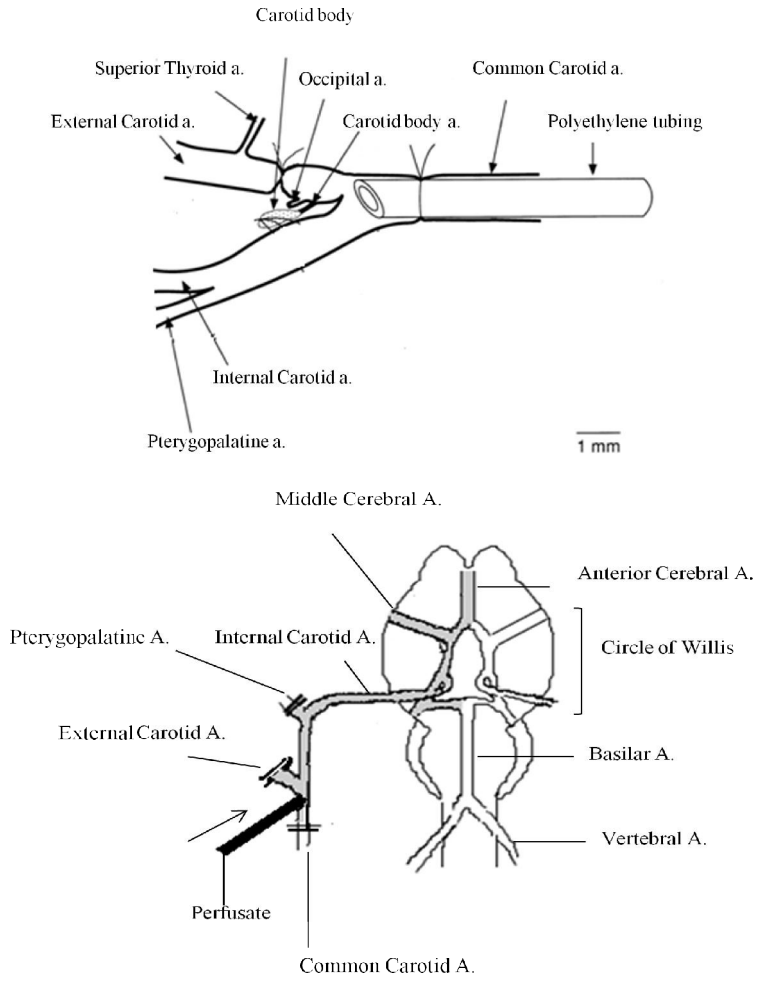


Figure 2.8. A schematic illustration of *in situ* rat brain perfusion technique modified from (Foster and Roberts, 2000). Perfusion medium is pumped into the brain capillaries through the common carotid artery.

2.4.4 *In vivo* methods

In vivo brain uptake studies can be divided into two approaches: the single pass uptake approach and the continuous uptake approach (Smith, 1989). The techniques using the single pass approach include the indicator diffusion technique and the brain uptake index technique and the single injection-external registration technique. The most commonly used continuous uptake approach is the intravenous administration technique. In the intravenous administration technique, the drug is injected intravenously as a bolus injection or as a constant infusion. The time range in which the samples are drawn from blood can range from 10 seconds to several hours, and at the end brain is removed for sampling. The intravenous technique remains the reference for other brain uptake studies because it represents fully physiological conditions. Moreover, it offers the possibility to measure tissue uptake over long periods of time. In addition, information on systemic pharmacokinetics of a compound may be gained simultaneously (Bickel, 2005). This is considered as one of the main advantages of this technique. In addition, the possibility to evaluate the pharmacokinetics of drugs in steady state adds the value of the intravenous injection method. However, there are potential pitfalls which should be noted in order to avoid erroneous evaluation and data interpretation. Especially with metabolically labile substances, specific analytical methods to measure the intact fraction in blood and tissue have to be developed. Furthermore, unless there is irreversible binding of the test substance in brain tissue, the movement across the BBB is bidirectional at sufficiently late time points. Moreover, the interpretation of the results can be difficult because of the complexity of the peripheral pharmacokinetics. Therefore, it can be problematic to evaluate some individual process involved in the brain uptake. In addition, when drug concentrations are determined from whole brain tissue, the unbound concentration of the drug in the brain parenchyma cannot be evaluated. Finally only one time point

can be determined from one experiment leading to sacrifice of many animals before sufficient data can be gathered.

Brain microdialysis

Intracerebral microdialysis involves direct sampling of brain ECF by implanting a dialysis probe into the brain. The microdialysis probe consists of a semipermeable membrane and is perfused with a physiological solution, whereby compounds that are small enough to traverse the semipermeable membrane diffuse from higher to lower concentration according to concentration gradient (Fig. 2.9) (de Lange et al., 1999). The concentration of compound that has permeated into the brain following oral, intravenous or subcutaneous administration can be monitored over time within the same animal. Therefore, one of the major advantages compared to whole brain concentration determination is that microdialysis provides pharmacokinetic profiles of compounds in the brain without the need to sacrifice many animals at different time points. In addition, with microdialysis, the free concentration in the ECF is measured, which is pharmacologically the more important fraction of the concentration than the non-specifically bound fraction (de Lange and Danhof, 2002). However, it cannot always be concluded that the ECF and ICF concentrations are equal. Therefore, when the site of action is located to be intracellular, the microdialysis and whole brain tissue data should be combined in order to evaluate the free ICF drug concentration (de Lange and Danhof, 2002). One major disadvantage of the microdialysis technique is that if one is not interested in localized concentrations, this raises the issue of where to place the probe and whether multiple probes should be used in order to optain an appropriate representation of drug levels throughout the brain (Bonate, 1995). The other major disadvantage associated with intracerebral microdialysis is that insertion of the probe can result in chronic BBB disruption (Westergren et al., 1995). In addition, valid quantitative interpretation of microdialysis data requires the individual

calibration of the probes to obtain correction factors, which relate the dialysate concentration to the brain ECF concentration. However, drug extraction across the probe membrane measured *in vitro* is generally greater than that occurring *in vivo*. Even though *in vitro* calibration of the probes is considered sufficient, *in vivo* recovery may still deviate from *in vitro* recovery (de Lange et al., 1999).

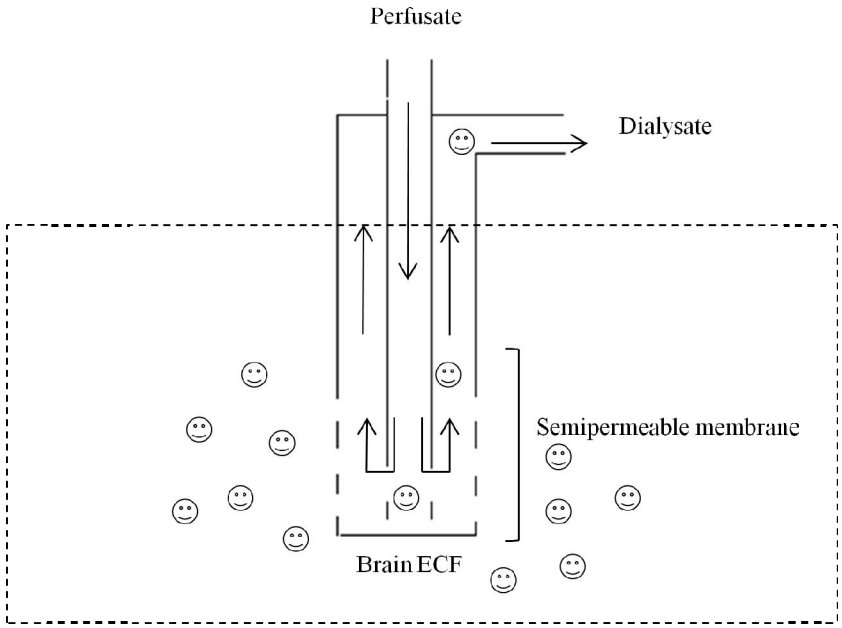


Figure 2.9. A schematic illustration of brain microdialysis.

2.5 METHODS FOR OVERCOMING THE POOR BRAIN UPTAKE OF DRUGS

Larger molecular weight and hydrophilic drugs tend to fail in the drug discovery process because of their poor ability to cross BBB. However, only a few brain diseases consistently respond to small lipid-soluble molecules (Ajay et al., 1999). Therefore, the development of new brain targeting strategies and their seamless integration into the drug design process is necessary (Pardridge, 2003). There are both chemistry-based and biology-

based approaches for developing new brain targeting strategies (Pardridge, 2001a). The chemistry-based strategies rely on passive diffusion of drugs across the BBB, whereas the biology-based strategies for brain drug delivery are based on the principle that there are numerous endogenous transport systems within the BBB, and these transporters are capable of transporting drug molecules into the brain.

2.5.1 Improving the physicochemical properties of drugs

The paracellular route across the BBB is efficiently restricted by tight junctions. Therefore, the physicochemical properties of drug molecules have to be optimal for transcellular uptake in order to enable the passive permeation across the BBB. These properties are mentioned in Table 2.1. A very simple approach to increase the CNS entry of polar molecules would be the masking of polar functionalities. This is sometimes referred to as the lipidization of molecules (Pardridge, 2007a). In practice, lipidization through lipophilic drug analogues often results in diminished therapeutic effect, due to decreased activity of the analogues or increased toxicity (Rautio et al., 2008). Lipidization through prodrugs offers a possibility for a more efficient CNS delivery of polar drugs without compromising their pharmacological effect or safety. Prodrugs, being more lipophilic than the parent drug, enter the CNS more readily, and are then converted back to the parent drug within the CNS (Anderson, 1996). However, lipophilic prodrugs share some problems with lipophilic drug analogues which limit the usefulness of the lipidization approach. Lipophilic modifications can lead to an increase in molecular weight of the drug, and the effect of molecular weight on molecule diffusion through biological membranes has been well documented. Increased lipid solubility not only increases permeation across the BBB, but also permeation across all other biological membranes in the body. Therefore, the increased BBB permeability is offset by increased plasma clearance and reduced AUC and there may be little increase in brain uptake of the drug, even though the BBB

permeability has been greatly enhanced. Increased lipid solubility of a drug may enhance drug binding to plasma proteins, which can lead to a low free plasma concentration of the drug. Since it is the concentration gradient of the free fraction of the drug that acts as the driving force across the BBB, the enhanced membrane permeation caused by lipid solubility can be offset by plasma protein binding. Probably the most important factor that limits the usefulness of lipidization is high non-specific brain tissue binding of lipophilic drugs. The enhanced brain-to-plasma ratio and brain concentration of lipophilic prodrugs or analogues is often due to non-specific brain tissue binding (Summerfield et al., 2006; Friden et al., 2007; Jeffrey and Summerfield, 2007). The optimization of drug brain uptake by improving the brain-to-plasma ratio has been the cornerstone of drug brain targeting in the past. However, as recent studies show, the optimization of this parameter is often futile, instead it is the free concentration in the brain that should be optimized (Jeffrey and Summerfield, 2007; Hammarlund-Udenaes et al., 2008; Liu et al., 2008). Despite several limitations, many lipidization prodrug strategies have been proposed as ways to achieve enhanced drug brain uptake (Oldendorf et al., 1972; Anderson, 1996; Rautio et al., 2008).

2.5.2 Carrier-mediated uptake

Several specific carrier-mediated transporters have been identified at the brain capillary endothelium that forms the BBB *e.g.* LAT, MCT1 and GluT1 (Pardridge and Oldendorf, 1977). As many drug molecules have similar structural properties to endogenous substrates, it is clear that some membrane transporters can also participate in drug transport (Tamai and Tsuji, 2000). In many cases it is pure coincidence that the drugs are able to utilize a transporter for BBB permeation. Moreover, making chemical drug modifications in such a way that the drug can be recognized by specific transporters, but still maintaining its therapeutic efficacy, has proven to be very challenging (Rautio et al., 2008). However, those drugs which

can utilize BBB transporters serve as good examples that the transporters present at the BBB are able to carry molecules other than their endogenous substrates.

One attractive approach to utilize BBB transporters is to conjugate an endogenous transporter substrate to the active drug molecule in a bioreversible manner; *i.e.*, to utilize the prodrug approach. The prodrug should be designed in such a way that it is recognized by the specific transporter mechanism at the BBB, and transported across the BBB to brain tissues, where the release of an active drug from the prodrug should predominantly take place. However, the CNS drug delivery via prodrugs can be compromised because of premature systemic bioconversion of the prodrug, although the structural requirements for transporter recognition are fulfilled. Moreover, the prodrugs have to compete with endogenous substrates for the transporters (del Amo et al., 2008). In addition, as the transporters located in the BBB may also be present in other tissues and there can be overlap of substrates with other transporters, the prodrug may be channeled also into other tissues. The plasma protein binding of highly bound drugs may decrease significantly due to hydrophilic nutrient promoiety (van de Waterbeemd et al., 2001). Therefore, the systemic pharmacokinetics may change e.g. the higher free concentration of drugs can increase the brain uptake due to increased concentration gradient. The addition of a nutrient promoiety to drug molecule often increases the hydrophilicity of the molecule. Therefore, the passive brain uptake may be decreased due to lower LogP (Fischer et al., 1998; Summerfield et al., 2007). High hydrophilicity of prodrugs often leads to a decrease in the non-specific brain tissue binding. Therefore, the free concentration of the drug in brain parenchyma may not be changed, even though the whole brain concentration is decreased (Jeffrey and Summerfield, 2007; Summerfield et al., 2007). The major advantage of nutrient mimicking prodrugs over lipophilic prodrugs or drug analogues is their ability to maintain BBB permeability without increasing lipophilicity. In addition, nutrient mimicking prodrugs may gain transporter

mediated access into the intracellular compartments of the brain. It has been postulated that the optimization of the CNS uptake should be integrated early into the drug discovery process. However, some brain targeting strategies, such as prodrug design can be utilized later in the drug discovery process, if the parent drug fails to cross the BBB sufficiently. The carrier-mediated transporter utilizing prodrug strategy has been studied most extensively for LAT1 and GluT1 systems (Walker, 1994; Halmos et al., 1996; Bonina et al., 1999; Bonina et al., 2003; Fernandez et al., 2003).

The utilization of LAT1 in brain drug delivery

LAT1 has been suggested to contribute in the transport of several clinically useful amino acid mimicking drugs, such as L-dopa, melphalan, baclofen, 3-O-methyldopa, alpha-methyltyrosine, gabapentin, alphamethyldopa, and thyroid hormones, thus demonstrating the ability of this transporter to be utilized in drug delivery (Cornford et al., 1992; Deguchi et al., 1995; Gomes and Soares-da-Silva, 1999a; Gomes and Soares-da-Silva, 1999b; Ritchie et al., 1999; Uchino et al., 2002). It should be not surprising that all of these drugs bear a very close structural resemblance to endogenous LAT1-substrates. The LAT1-mediated brain uptake of these drugs seems to be a coincidence and they were not designed to utilize LAT1. Although, there are these examples of LAT1 utilizing drugs, the design of novel drugs that are able to cross the BBB via LAT1 and still maintain their therapeutic activity might be difficult. Therefore, a different approach to utilize LAT1 has been suggested, such as the conjugation of drug molecules with amino acids by a bioreversible bond *i.e.* by using a prodrug approach (Walker, 1994; Killian et al., 2007).

Drug molecules can be conjugated with amino acids that bear a functional group suitable for prodrug bond in their side chain. The conjugation from the side chain leaves the functional groups necessary for the LAT1 affinity available. Once the prodrug has crossed the BBB it needs to release the parent drug.

Evidence acquired by determining the affinity of four melphalan analogues suggests the binding affinity for LAT1 is dependent not only upon side chain hydrophobicity but upon the 3D structure of the side chain (Fig. 2.10) (Smith, 2005). It seems that the meta-position of the side chain is the most favourable position to attain high affinity for LAT1. Moreover, there is some evidence that the maximal transport velocity decreases with the substrate size. The effect of sterical hindrance on the maximum uptake velocity was elegantly shown in the study of (Takada et al., 1992). The affinity, transport velocity and PA product of melphalan and melphalan analogoues were determined with *in situ* rat brain perfusion technique. One melphalan analogue, DL-NAM, showed higher affinity for LAT1 and maximum PA product compared to melphalan. In addition, the K_m of DL-NAM has significantly lower compared to K_m of L-phenylalanine and L-leucine. However, the maximum transport velocity *in situ* was 20 times lower compared to melphalan and 150 times lower compared to L-phenylalanine in *in situ* perfusion experiments. This indicates that though, DL-NAM does bind to LAT1 with high affinity, the presence of a bulky side chain leads to decreased transport velocity and the LAT1-mediated uptake becomes saturated at low concentrations (Takada et al., 1992). Despite the low uptake velocity, DL-NAM is an excellent candidate for LAT1-mediated brain uptake and serves as good example that LAT1 can be utilized for CNS drug delivery. DL-NAM has higher affinity for LAT1 than the endogenous substrates and therefore its plasma concentration can remain low and still achieve LAT1-mediated brain uptake. In addition, high affinity for LAT1 and high maximum PA product indicates that DL-NAM is efficiently transported into cells which express LAT1, such as brain cells and some cancer cells (Takada et al., 1992).

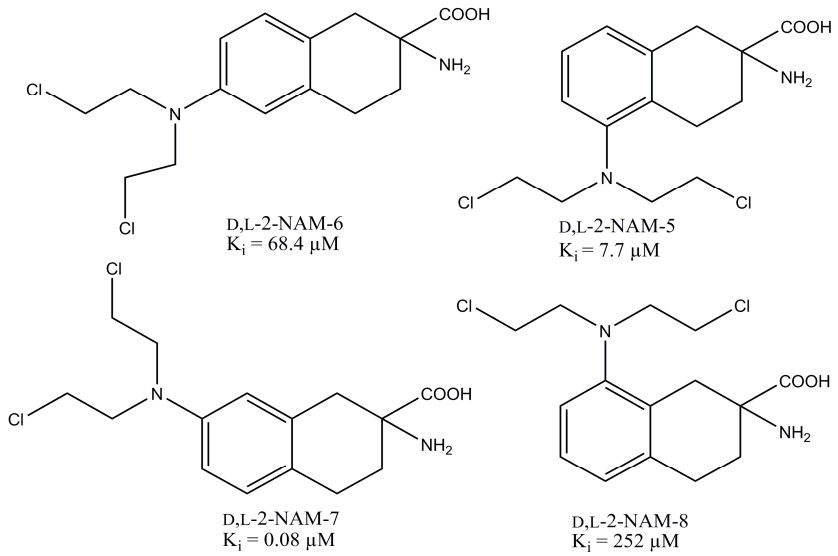


Figure 2.10. Structures and K_i values for LAT1 binding of four positional isomers of DL-NAM with the nitrogen mustard in either the ortho (DL-2-NAM-8), meta (DL-2-NAM-5,-7) or para (DL-2-NAM-6) positions (Smith, 2005).

LAT1 exhibits poor affinity for some amino acids is due to hydrophilic functional group in the side chain. This functional group can be used to link the parent drug with the amino acid and as the hydrophilic functional group is masked from the side chain, the resulting prodrug may have good affinity for LAT1. This approach enlarges the group of possible amino acids that can be utilized. In addition, it is possible to design and synthesize new amino acid mimicking molecules, which can be used as promoieties. However, natural amino acids have some advantages compared to their synthetic counterparts. Natural amino acids are cheap and readily available. Furthermore, the effects of natural amino acids on human body are known, and when using natural amino acids, the risk of side effects in the brain caused by the released amino acids is lower compared to synthetic promoieties.

There are certain limitations to the utilization of LAT1. LAT1 transporters in the BBB are normally saturated by the amino acids present in plasma. The total concentration of relevant

amino acids in plasma ranges from 0.4 mM to 2.3 mM, and their average inhibition constant (K_i) to LAT1 is about 70–100 μM (Huang et al., 1998). Therefore, in order to utilize the LAT1 system, the drugs and prodrugs have to compete with plasma amino acids, and the affinity of the compounds for LAT1 has to be high. In a recent review, it was reported that LAT1-mediated brain uptake of L-dopa and melphalan is limited because LAT1 is saturated (del Amo et al., 2008). The LAT1-mediated L-dopa and melphalan transport across the BBB is reduced by 98-99.6% and 98.7-99.7% by 400-2300 μM concentration of amino acids, respectively. However, as was shown with DL-NAM, molecules with higher affinities than endogenous LAT1 substrates can be designed. The drugs should also cross the abluminal membrane, and as there is high surplus of amino acids in the cytosol compared to the drug, most probably only a minor fraction of the efflux function of LAT1 is used by the drugs (del Amo et al., 2008). Furthermore the putative Na^+- dependent large neutral amino acid transporter may pump drugs from the brain to the blood circulation (Hawkins et al., 2005). LAT1 and other amino acid transporters are also expressed in tissues other than the BBB. This may lead to accumulation of the substrates into these tissues. With systemic administration, the prodrug bonds between the parent drug and amino acids are most likely susceptible to degradation by enzymes which are present in many tissues, not solely in the brain parenchyma. This can lead to the premature release of the parent drug in peripherial tissues. As the brain uptake of drugs is efficiently limited by the BBB, even small improvement of brain uptake may be adequate.
Although L-dopa has been in clinical use for more than 40 years, there are not many drugs in clinical use or published prodrugs that utilize LAT1 for brain uptake (Pardridge, 2003). This may be due to poor understanding of the limitations and possibilities of LAT1 utilization. It seems that LAT1 utilization could be most useful for small molecular weight drugs, which are not able to cross the BBB because of their hydrophilicity. In addition, drugs with high plasma protein binding may benefit from this prodrug strategy. With an amino acid promoiety, it may be

possible to enhance the brain uptake of hydrophilic drugs without increasing the lipophilicity. Therefore, the non-specific binding to brain tissue and the increased distribution into other tissues than brain should be avoided. In addition, the presence of amino acid transporters at the brain parenchyma may increase the cellular uptake of amino acid prodrugs, which would be useful, if the target protein of the parent drug is located inside the cell (Thurlow et al., 1996; Simpson et al., 2007). Gapapentin has been shown to have higher brain distribution volume than its lipophilicity would indicate, and it has been concluded that the cellular uptake of gabapentin is carrier-mediated (Friden et al., 2007). In fact it has been proposed that the gabapentin uptake into astrocytes and synaptosomes is rapid, and the uptake is mediated by the same transporter as the uptake of L-leucine (Su et al., 1995; Wang and Welty, 1996). LAT1 is a good option for a carrier-mediated transporter targeting strategy, because there are several examples that compounds other than LAT1 endogenous substrates are able to utilize this transporter to cross the BBB. In addition, the rational design of LAT1 substrates is possible, because there is understanding of what the structural properties make molecules LAT1 substrates. Furthermore, amino acids have suitable functional groups which can be utilized for the formation of prodrug bond between the amino acid and drug molecule.

Examples of prodrugs that have been designed to utilize LAT1 for brain uptake

The studies published so far involving LAT1-mediated brain uptake of prodrugs are LAT1 substrate uptake inhibition studies. These studies are a simple and quick way to determine the ability of the prodrug to inhibit the uptake of endogenous substrate, and therefore, demonstrate its ability to bind to LAT1. However, these studies do not provide any insight into whether the prodrugs were able to cross the BBB. For example, L-cysteine was conjugated with the anticancer agent 6-mercaptopurine (Fig. 2.11) and a model compound 2-methyl-1-propanethiol

(Killian et al., 2007). The prodrugs were able to inhibit LAT1-mediated brain uptake of [^{14}C]L-leucine using an *in situ* rat brain perfusion technique, which indicated that the prodrugs are able to bind to LAT1. It has been reported that an antiviral agent phosphonoformate L-tyrosine conjugate could inhibit the transport of [^{3}H]L-tyrosine in porcine brain microvessel endothelial cells (Fig 2.11) (Walker, 1994). In another study, *p*-nitro and *p*-chlorobenzyl ether conjugates of L-tyrosine inhibited the transport of [^{3}H]L-tyrosine in rabbit corneal cell line (Fig. 2.11) (Balakrishnan et al., 2002). These results indicate that L-tyrosine and L-cysteine conjugates are able to bind to the LAT1-transporter. However, the ability of these conjugates to cross the cell membranes has not been evaluated. Moreover, the *in vivo* brain uptake of these conjugates cannot be estimated from the *in situ* brain perfusion and *in vitro* results. However, there is an example of *in vivo* study on amino acid prodrugs. The anticonvulsant activity of nipecotic acid L-tyrosine ester was studied in an *in vivo* epilepsy rat model (Fig. 2.11) (Bonina et al., 1999). The prodrug was able to reach therapeutically active concentrations in the CNS. However, the mechanism of brain uptake remains unclear as the prodrug is very lipophilic and no further BBB transport studies were conducted for this prodrug. In addition to BBB permeation, the conjugates should release the parent drug in the brain parenchyma and be stable in the systemic circulation. Some estimation of the prodrug stability can be made with *in vitro* tissue homogenate studies. However, *in vivo* studies need to be conducted before any conclusions of the stability can be made.

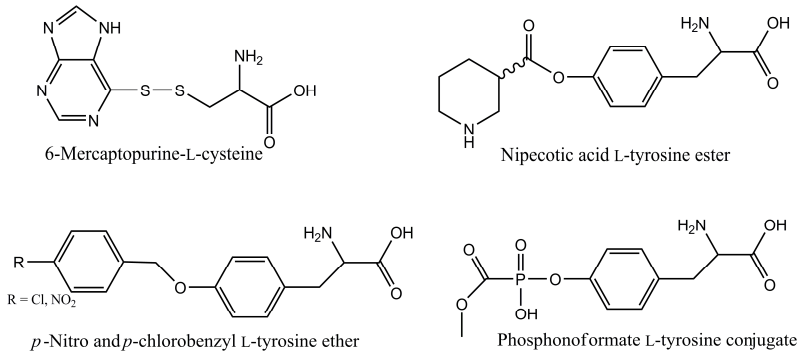


Figure 2.11. Structures of amino acid conjugates tested *in vivo*, *in vitro* or *in situ* (Walker, 1994; Bonina et al., 1999; Balakrishnan et al., 2002; Killian et al., 2007).

The utilization of GluT1 in brain drug delivery

GluT1 is present both on the luminal and the abluminal membranes of the endothelial cells making up the BBB (Farrell and Pardridge, 1991). GluT1 transports glucose and other hexoses, and has the highest transport capacity of all the carrier-mediated transporters present at the BBB, being therefore an attractive transporter for prodrug delivery (Anderson, 1996). The utilization of GluT1 for carrier-mediated brain uptake has been studied with several techniques (Battaglia et al., 2000; Fernandez et al., 2003; Garcia-Alvarez et al., 2007). However, the results from these studies are somewhat inconsistent. *In vitro* studies have revealed that glycosyl conjugates bind to GluT1 and inhibit the uptake of substrates like glucose. Moreover, some structure activity relationship data are available, which are partially consistent with a model for the exofacial configuration and the substrate binding site of GluT1 (Fernandez et al., 2003; Mueckler and Makepeace, 2008). There are also data that show that some of the conjugates that bind to GluT1 are not able to cross the cell membrane via GluT1 and they act merely as glucose uptake inhibitors (Halmos et al., 1996; Garcia-Alvarez et al., 2007). Results from *in vivo* experiments demonstrated that glucose conjugates were able to cross the BBB *in vivo* and release the parent drug in pharmacologically active amounts in the CNS

(Fig. 2.13) (Bonina et al., 1999; Battaglia et al., 2000; Bonina et al., 2003). However, the mechanism of the brain uptake remains unclear. In addition, the bioreversible bond between the parent drug and glucose is often labile, which limits the use of these prodrugs. Moreover, there is a risk involved when GluT1 is utilized for brain uptake. The efficient brain uptake of glucose is essential to maintain normal cerebral function and thus the supply of glucose uptake could be disrupted, if GluT1 is utilized via prodrugs. However, GluT1 utilization is still a potential strategy for enhanced brain uptake of prodrugs. In particular, the brain uptake of small hydrophilic drug molecules could be enhanced by inclusion of a glucose promoiety. The vast transport capacity and the poor affinity of the endogenous substrate glucose can be possibly utilized with good prodrug design. If the affinity of the prodrug for GluT1 is much higher than the affinity of glucose, advantage could be made of the vast capacity of GluT1 to handle low plasma drug concentrations. In addition, the sufficient stability of the glucose prodrugs needs to be addressed, if new prodrugs are designed for per oral drug administration.

Examples of prodrugs that have been designed to utilize GluT1 for brain uptake

The glycosylation strategy has been utilized in an attempt to increase the brain uptake of several CNS active drugs, such as dopamine, chlorambucil, 7-chlorokynurenic acid and GW196771 (Fig. 2.12 and 2.13) (Halmos et al., 1996; Battaglia et al., 2000; Fernandez et al., 2000; Bonina et al., 2003; Angusti et al., 2005). The studies considering the uptake mechanism of glucose prodrugs have been performed *in vitro* by determining the ability of the glycosyl conjugates to inhibit the uptake of radiolabeled substrate. In addition, some *in vitro* studies also include the determination of the cellular uptake of the prodrugs. A glucose–chlorambucil derivative was able to inhibit the uptake of [^{14}C]D-glucose into human erythrocytes (Halmos et al., 1996). However, in these *in vitro* uptake studies, the prodrug

was found to be an inhibitor rather than a substrate of GluT1. Several glycosyl derivatives of dopamine were synthesized and tested for the affinity of the prodrugs for GluT1 in human erythrocytes (Fernandez et al., 2003). Dopamine was linked to glucose with different linkers at the C-1, C-3 and C-6 positions of glucose (Fig. 2.12). The results of glucose uptake inhibition revealed that the glucose derivatives that were conjugated at position C-6 had the best affinity for GluT1. There was also a difference in the affinity when carbamate or succinamate was used as linkers between dopamine and the promoiety, with the carbamate prodrug having better affinity for the carrier. In addition, human retinal pigment epithelium cells were used to determine the cellular uptake mechanism of the dopamine-glucose conjucate (Dalpiaz et al., 2007). The prodrug was obtained by coupling dopamine to D-glucose via a succinic spacer from the C-3 position. The result suggested that the dopamine prodrug was able to inhibit the uptake of [^{3}H]3-O-methylglucose in a concentration-dependent manner. In addition, it was observed that the dopamine prodrug was able to permeate into the cells, and the uptake of the prodrug was significantly inhibited in the presence of 10 mM D-glucose. The cellular uptake of several glucose conjugates of dopamine derivatives into erythrocytes was determined in the presence and absence of GluT1 inhibitors (Garcia-Alvarez et al., 2007). The dopamine derivatives were conjugated with glucose at the C-6 position based on the earlier studies of the research group, indicating that conjugation at C-6 position resulted in the best affinity for GluT1. There was a difference in the uptake rate of the glucose-dopamine derivative conjugates into erythrocytes. These results indicated that hydrophilic derivatives displayed the poorest uptake into the cells. In addition, two GluT1 inhibitors, maltosyl isothiocyanate and cytochalasin B, were not able to inhibit the uptake, indicating that the cellular uptake of the conjugates was not GluT1-mediated.

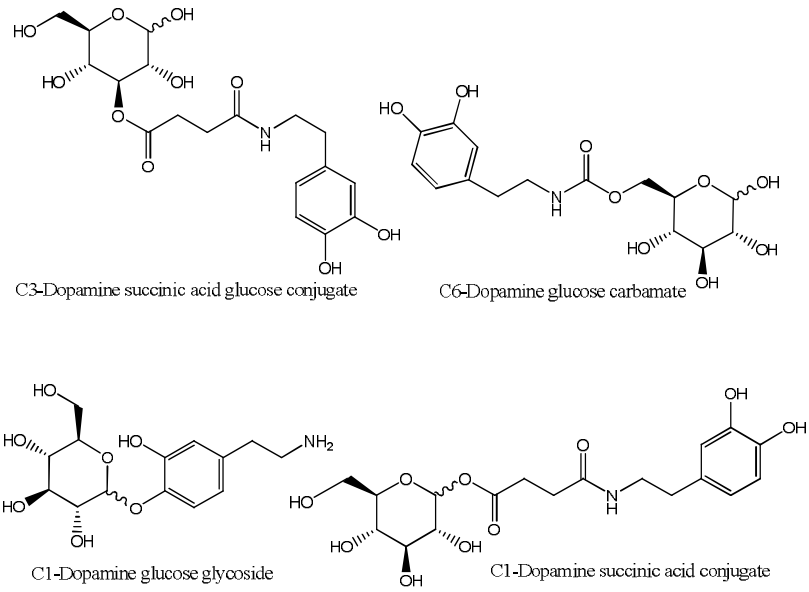


Figure 2.12. Examples of dopamine-glucose prodrugs (Fernandez et al., 2003; Dalpiaz et al., 2007).

Some studies with glucose conjugates have been performed in mice and rats *in vivo*. Glycosyl derivatives of dopamine that were conjugated with a succinyl linker did not exhibit any ability to induce recovery of the motor activity of mice pretreated with reserpine (Fernandez et al., 2000). This was attributed to slow the bioconversion rate of the prodrugs into dopamine. In another study L-dopa and dopamine glycoside prodrugs were synthesized by conjugating the parent drugs with glucose at the C-3 position and galactose at the C-6 position by a succinyl linker with the aim of overcoming the problem of the low BBB permeability of dopamine (Bonina et al., 2003). The prodrugs were tested with classic dopaminergic models, morphine induced locomotion in mice and reserpine-induced hypolocomotion in rats. Both of the dopamine glycosidic prodrugs were more active in reversing the reserpine-induced hypolocomotion in rats than L-dopa or the L-dopa prodrugs. For example, in reducing morphine-induced locomotion in mice, the galactose-dopamine conjugate was the most effective and glucose-dopamine had the lowest efficacy of

all the tested prodrugs. However, the glucose-L-dopa prodrug was more effective than the galactose-L-dopa prodrug. Thus, it seems that by conjugating dopamine with glycosides, the pharmacological efficacy had been increased but the mechanism of brain uptake remained unclear. In other words, the increased pharmacological activity of galactose-dopamine conjugate might not be due to GluT1-mediated increased brain permeability. The concentration of 7-chlorokynurenic acid and kynurenic acid in the rat brain was determined with microdialysis after systemic injection of 7-chlorokynurenic acid with two glucose conjugates of 7-chlorokynurenic acid (Fig. 2.13) (Battaglia et al., 2000). The glycosylation increased the brain uptake of the parent drug, but the mechanism responsible for the increased uptake was unfortunately not conclusively demonstrated in that study.

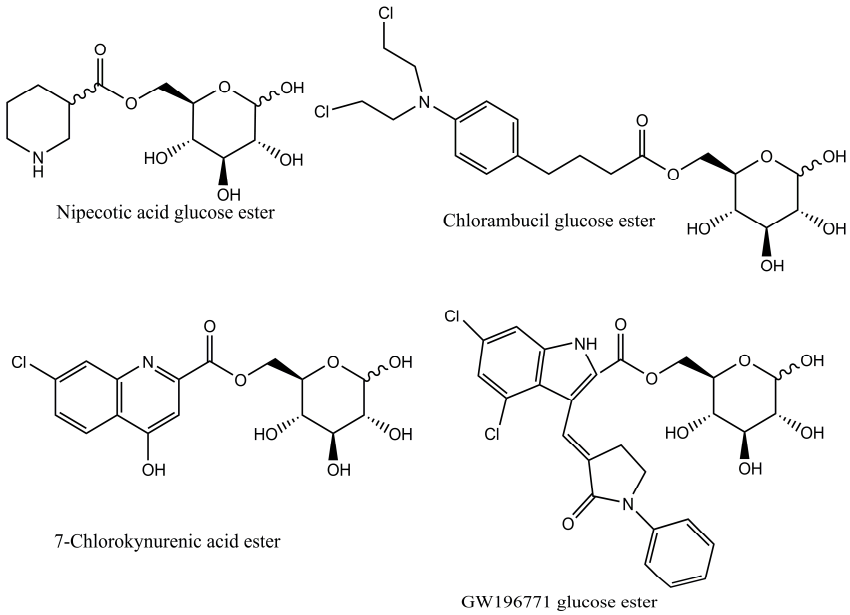


Figure 2.13. The structures of D-glucose prodrugs tested *in vivo* (Halmos et al., 1996; Bonina et al., 1999; Battaglia et al., 2000; Angusti et al., 2005).

2.6 REFERENCES

Abbott N: Astrocyte-endothelial interactions and blood-brain barrier permeability. J Anat 200: 527, 2002.

Ajay, Bemis G W, Murcko M A: Designing libraries with CNS activity. J Med Chem 42: 4942-4951, 1999.

Allt G and Lawrenson J G: Pericytes: cell biology and pathology. Cells Tissues Organs 169: 1-11, 2001.

Amann T, Maegdefrau U, Hartmann A, Agaimy A, Marienhagen J, Weiss T S, Stoeltzing O, Warnecke C, Scholmerich J, Oefner P J, Kreutz M, Bosserhoff A K, Hellerbrand C: GLUT1 expression is increased in hepatocellular carcinoma and promotes tumorigenesis. Am J Pathol 174: 1544-1552, 2009.

Anderson B D: Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19: 171-202, 1996.

Angusti A, Durini E, Vertuani S, Dalpiaz A, Ruffo A, Di Fabio R, Donati D, Pentassuglia G, Vitulli G, Barnaby R J, Manfredini S: Synthesis and biological evaluation of pro-drugs of GW196771, a potent glycine antagonist acting at the NMDA receptor. Farmaco 60: 393-397, 2005.

Balakrishnan A, Jain-Vakkalagadda B, Yang C, Pal D, Mitra A K: Carrier mediated uptake of L-tyrosine and its competitive inhibition by model tyrosine linked compounds in a rabbit corneal cell line (SIRC)--strategy for the design of transporter/receptor targeted prodrugs. Int J Pharm 247: 115-125, 2002.

Ballabh P, Braun A, Nedergaard M: The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 16: 1-13, 2004.

Battaglia G, La Russa M, Bruno V, Arenare L, Ippolito R, Copani A, Bonina F, Nicoletti F: Systemically administered D-glucose conjugates of 7-chlorokynurenic acid are centrally available and exert anticonvulsant activity in rodents. Brain Res 860: 149-156, 2000.

Beck D W, Roberts R L, Olson J J: Glial cells influence membrane-associated enzyme activity at the blood-brain barrier. Brain Res 381: 131-137, 1986.

Begley D J: ABC transporters and the blood-brain barrier. Curr Pharm Des 10: 1295-1312, 2004a.

Begley D J: Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 104: 29-45, 2004b.

Begley D J and Brightman M W: Structural and functional aspects of the blood-brain barrier. Prog Drug Res 61: 39-78, 2003.

Begley D J, Lechardeur D, Chen Z D, Rollinson C, Bardoul M, Roux F, Scherman D, Abbott N J: Functional expression of P-glycoprotein in an immortalised cell line of rat brain endothelial cells, RBE4. J Neurochem 67: 988-995, 1996.

Betz A L, Firth J A, Goldstein G W: Polarity of the blood-brain barrier: distribution of enzymes between the luminal and antiluminal membranes of brain capillary endothelial cells. Brain Res 192: 17-28, 1980.

Bickel U: How to measure drug transport across the blood-brain barrier. NeuroRx 2: 15-26, 2005.

Bickel U, Kang Y S, Yoshikawa T, Pardridge W M: In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. J Histochem Cytochem 42: 1493-1497, 1994.

Bickel U, Yoshikawa T, Pardridge W M: Delivery of peptides and proteins through the blood-brain barrier. Adv Drug Deliv Rev 46: 247-279, 2001.

Birnbaum M J, Haspel H C, Rosen O M: Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. Proc Natl Acad Sci U S A 83: 5784-5788, 1986.

Blodgett D M and Carruthers A: Quench-flow analysis reveals multiple phases of GluT1-mediated sugar transport. Biochemistry 44: 2650-2660, 2005.

Boado R J, Li J Y, Chu C, Ogoshi F, Wise P, Pardridge W M: Site-directed mutagenesis of cysteine residues of large neutral amino acid transporter LAT1. Biochim Biophys Acta 1715: 104-110, 2005.

Boado R J, Li J Y, Nagaya M, Zhang C, Pardridge W M: Selective expression of the large neutral amino acid transporter at the blood-brain barrier. Proc Natl Acad Sci U S A 96: 12079-12084, 1999.

Bonate P L: Animal models for studying transport across the blood-brain barrier. J Neurosci Methods 56: 1-15, 1995.

Bonina F, Puglia C, Rimoli M G, Melisi D, Boatto G, Nieddu M, Calignano A, La Rana G, De Caprariis P: Glycosyl derivatives of dopamine and L-dopa as anti-Parkinson prodrugs: synthesis, pharmacological activity and in vitro stability studies. J Drug Target 11: 25-36, 2003.

Bonina F P, Arenare L, Palagiano F, Saija A, Nava F, Trombetta D, de Caprariis P: Synthesis, stability, and pharmacological evaluation of nipecotic acid prodrugs. J Pharm Sci 88: 561-567, 1999.

Borst P, Evers R, Kool M, Wijnholds J: A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 92: 1295-1302, 2000.

Braun L D, Cornford E M, Oldendorf W H: Newborn rabbit blood-brain barrier is selectively permeable and differs substantially from the adult. J Neurochem 34: 147-152, 1980.

Brightman M W and Kaya M: Permeable endothelium and the interstitial space of brain. Cell Mol Neurobiol 20: 111-130, 2000.

Brown V I and Greene M I: Molecular and cellular mechanisms of receptor-mediated endocytosis. DNA Cell Biol 10: 399-409, 1991.

Bruni J E: Ependymal development, proliferation, and functions: a review. Microsc Res Tech 41: 2-13, 1998.

Chishty M, Begley D J, Abbott N J, Reichel A: Interaction of nucleoside analogues with nucleoside transporters in rat brain endothelial cells. J Drug Target 12: 265-272, 2004.

Cordon-Cardo C, O'Brien J P, Casals D, Rittman-Grauer L, Biedler J L, Melamed M R, Bertino J R: Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proc Natl Acad Sci U S A 86: 695-698, 1989.

Cornford E M and Hyman S: Localization of brain endothelial luminal and abluminal transporters with immunogold electron microscopy. NeuroRx 2: 27-43, 2005.

Cornford E M, Young D, Paxton J W, Finlay G J, Wilson W R, Pardridge W M: Melphalan penetration of the blood-brain barrier via the neutral amino acid transporter in tumor-bearing brain. Cancer Res 52: 138-143, 1992.

Cucullo L, Couraud P O, Weksler B, Romero I A, Hossain M, Rapp E, Janigro D: Immortalized human brain endothelial cells and flow-based vascular modeling: a marriage of convenience for rational neurovascular studies. J Cereb Blood Flow Metab 28: 312-328, 2008.

Dallas S, Miller D S, Bendayan R: Multidrug resistance-associated proteins: expression and function in the central nervous system. Pharmacol Rev 58: 140-161, 2006.

Dalpiaz A, Filosa R, de Caprariis P, Conte G, Bortolotti F, Biondi C, Scatturin A, Prasad P D, Pavan B: Molecular mechanism involved in the transport of a prodrug dopamine glycosyl conjugate. Int J Pharm 336: 133-139, 2007.

de Boer A G and Gaillard P J: Drug targeting to the brain. Annu Rev Pharmacol Toxicol 47: 323-355, 2007.

de Boer A G v d S, I. C.; Gaillard, P. J.: The role of drug transporters at the blood-brain barrier. Annual Review of Pharmacology and Toxicology 43: 629-656, 2003.

de Graaf R A, Pan J W, Telang F, Lee J H, Brown P, Novotny E J, Hetherington H P, Rothman D L: Differentiation of glucose transport in human brain gray and white matter. J Cereb Blood Flow Metab 21: 483-492, 2001.

de Lange E C and Danhof M: Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin Pharmacokinet 41: 691-703, 2002.

de Lange E C, de Boer B A, Breimer D D: Microdialysis for pharmacokinetic analysis of drug transport to the brain. Adv Drug Deliv Rev 36: 211-227, 1999.

de Lange E C M R, P.G.M.; Groenendaal, D.; van Steeg, T.J.: Toward the Prediction of CNS Drug-Effect Profiles in Physiological and

Pathological Conditions Using Microdialysis and Mechanism-Based Pharmacokinetic-Pharmacodynamic Modeling. AAPS Journal 7: E532-E543, 2005.

Deguchi Y, Inabe K, Tomiyasu K, Nozawa K, Yamada S, Kimura R: Study on brain interstitial fluid distribution and blood-brain barrier transport of baclofen in rats by microdialysis. Pharm Res 12: 1838-1844, 1995.

del Amo E M, Urtti A, Yliperttula M: Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. Eur J Pharm Sci 35: 161-174, 2008.

Di L, Kerns E H, Bezar I F, Petusky S L, Huang Y: Comparison of blood-brain barrier permeability assays: in situ brain perfusion, MDR1-MDCKII and PAMPA-BBB. J Pharm Sci 98: 1980-1991, 2009.

Dick A P, Harik S I, Klip A, Walker D M: Identification and characterization of the glucose transporter of the blood-brain barrier by cytochalasin B binding and immunological reactivity. Proc Natl Acad Sci U S A 81: 7233-7237, 1984.

Dobson P D and Kell D B: Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 7: 205-220, 2008.

Dohgu S, Takata F, Yamauchi A, Nakagawa S, Egawa T, Naito M, Tsuruo T, Sawada Y, Niwa M, Kataoka Y: Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. Brain Res 1038: 208-215, 2005.

Duelli R, Enerson B E, Gerhart D Z, Drewes L R: Expression of large amino acid transporter LAT1 in rat brain endothelium. J Cereb Blood Flow Metab 20: 1557-1562, 2000.

Duffy K R and Pardridge W M: Blood-brain barrier transcytosis of insulin in developing rabbits. Brain Res 420: 32-38, 1987.

El Hafny B, Chappey O, Piciotti M, Debray M, Boval B, Roux F: Modulation of P-glycoprotein activity by glial factors and retinoic acid in an immortalized rat brain microvessel endothelial cell line. Neurosci Lett 236: 107-111, 1997.

Engelhardt B: Development of the blood-brain barrier. Cell Tissue Res 314: 119-129, 2003.

Ennis S R, Kawai N, Ren X D, Abdelkarim G E, Keep R F: Glutamine uptake at the blood-brain barrier is mediated by N-system transport. J Neurochem 71: 2565-2573, 1998.

Farrell C L and Pardridge W M: Blood-brain barrier glucose transporter is asymmetrically distributed on brain capillary endothelial lumenal and ablumenal membranes: an electron microscopic immunogold study. Proc Natl Acad Sci U S A 88: 5779-5783, 1991.

Fatehi M I, Gerhart D Z, Myers T G, Drewes L R: Characterization of the blood-brain barrier: glycoconjugate receptors of 14 lectins in canine brain, cultured endothelial cells, and blotted membrane proteins. Brain Res 415: 30-39, 1987.

Fernandez C, Nieto O, Fontenla J A, Rivas E, de Ceballos M L, Fernandez-Mayoralas A: Synthesis of glycosyl derivatives as dopamine prodrugs: interaction with glucose carrier GLUT-1. Org Biomol Chem 1: 767-771, 2003.

Fernandez C, Nieto O, Rivas E, Montenegro G, Fontenla J A, Fernandez-Mayoralas A: Synthesis and biological studies of glycosyl dopamine derivatives as potential antiparkinsonian agents. Carbohydr Res 327: 353-365, 2000.

Fischer H, Gottschlich R, Seelig A: Blood-brain barrier permeation: molecular parameters governing passive diffusion. J Membr Biol 165: 201-211, 1998.

Forster C: Tight junctions and the modulation of barrier function in disease. Histochem Cell Biol 130: 55-70, 2008.

Foster K A and Roberts M S: Experimental methods for studying drug uptake in the head and brain. Curr Drug Metab 1: 333-356, 2000.

Frey A, Meckelein B, Weiler-Guttler H, Mockel B, Flach R, Gassen H G: Pericytes of the brain microvasculature express gamma-glutamyl transpeptidase. Eur J Biochem 202: 421-429, 1991.

Friden M, Gupta A, Antonsson M, Bredberg U, Hammarlund-Udenaes M: In vitro methods for estimating unbound drug concentrations in the brain interstitial and intracellular fluids. Drug Metab Dispos 35: 1711-1719, 2007.

Friedrich A, Prasad P D, Freyer D, Ganapathy V, Brust P: Molecular cloning and functional characterization of the OCTN2 transporter at the RBE4 cells, an in vitro model of the blood-brain barrier. Brain Res 968: 69-79, 2003.

Fromm M F: P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. Int J Clin Pharmacol Ther 38: 69-74, 2000.

Ganapathy V, Thangaraju M, Prasad P D: Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond. Pharmacol Ther 121: 29-40, 2009.

Garcia-Alvarez I, Garrido L, Fernandez-Mayoralas A: Studies on the uptake of glucose derivatives by red blood cells. ChemMedChem 2: 496-504, 2007.

Gerhart D Z, Enerson B E, Zhdankina O Y, Leino R L, Drewes L R: Expression of monocarboxylate transporter MCT1 by brain endothelium and glia in adult and suckling rats. Am J Physiol 273: E207-213, 1997.

Ghose A K, Viswanadhan V N, Wendoloski J J: A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. J Comb Chem 1: 55-68, 1999.

Golden P L, Maccagnan T J, Pardridge W M: Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. J Clin Invest 99: 14-18, 1997.

Goldstein G W: Endothelial cell-astrocyte interactions. A cellular model of the blood-brain barrier. Ann N Y Acad Sci 529: 31-39, 1988.

Gomes P and Soares-da-Silva P: Interaction between L-DOPA and 3-O-methyl-L-DOPA for transport in immortalised rat capillary cerebral endothelial cells. Neuropharmacology 38: 1371-1380, 1999a.

Gomes P and Soares-da-Silva P: L-DOPA transport properties in an immortalised cell line of rat capillary cerebral endothelial cells, RBE 4. Brain Res 829: 143-150, 1999b.

Gumbleton M and Audus K L: Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. J Pharm Sci 90: 1681-1698, 2001.

Halmos T, Santarromana M, Antonakis K, Scherman D: Synthesis of glucose-chlorambucil derivatives and their recognition by the human GLUT1 glucose transporter. Eur J Pharmacol 318: 477-484, 1996.

Hammarlund-Udenaes M, Friden M, Syvanen S, Gupta A: On the rate and extent of drug delivery to the brain. Pharm Res 25: 1737-1750, 2008.

Hammarlund-Udenaes M, Paalzow L K, de Lange E C: Drug equilibration across the blood-brain barrier--pharmacokinetic considerations based on the microdialysis method. Pharm Res 14: 128-134, 1997.

Hawkins R A, Mokashi A, Simpson I A: An active transport system in the blood-brain barrier may reduce levodopa availability. Exp Neurol 195: 267-271, 2005.

Hayashi Y, Nomura M, Yamagishi S, Harada S, Yamashita J, Yamamoto H: Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. Glia 19: 13-26, 1997.

Huang S C, Stout D B, Yee R E, Satyamurthy N, Barrio J R: Distribution volume of radiolabeled large neutral amino acids in brain tissue. J Cereb Blood Flow Metab 18: 1288-1293, 1998.

Inano A, Sai Y, Nikaido H, Hasimoto N, Asano M, Tsuji A, Tamai I: Acetyl-L-carnitine permeability across the blood-brain barrier and involvement of carnitine transporter OCTN2. Biopharm Drug Dispos 24: 357-365, 2003.

Jefferies W A, Brandon M R, Hunt S V, Williams A F, Gatter K C, Mason D Y: Transferrin receptor on endothelium of brain capillaries. Nature 312: 162-163, 1984.

Jeffrey P and Summerfield S G: Challenges for blood-brain barrier (BBB) screening. Xenobiotica 37: 1135-1151, 2007.

Joo F: The blood-brain barrier in vitro: the second decade. Neurochem Int 23: 499-521, 1993.

Jordan F L and Thomas W E: Brain macrophages: questions of origin and interrelationship. Brain Res 472: 165-178, 1988.

Kalvass J C and Maurer T S: Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. Biopharm Drug Dispos 23: 327-338, 2002.

Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E, Endou H: Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J Biol Chem 273: 23629-23632, 1998.

Keep R F and Jones H C: A morphometric study on the development of the lateral ventricle choroid plexus, choroid plexus capillaries and ventricular ependyma in the rat. Brain Res Dev Brain Res 56: 47-53, 1990.

Killian D M, Hermeling S, Chikhale P J: Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. Drug Deliv 14: 25-31, 2007.

Kusuhara H and Sugiyama Y: Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 1). Drug Discov Today 6: 150-156, 2001.

Lai C H and Kuo K H: The critical component to establish in vitro BBB model: Pericyte. Brain Res Brain Res Rev 50: 258-265, 2005.

LaManna J C and Harik S I: Regional comparisons of brain glucose influx. Brain Res 326: 299-305, 1985.

Langen K J, Bonnie R, Muhlensiepen H, Jansen P, Broer S, Holschbach M, Coenen H H: 3-[123I]iodo-alpha-methyl-

L-tyrosine transport and 4F2 antigen expression in human glioma cells. Nucl Med Biol 28: 5-11, 2001.

Larson D M, Carson M P, Haudenschild C C: Junctional transfer of small molecules in cultured bovine brain microvascular endothelial cells and pericytes. Microvasc Res 34: 184-199, 1987.

Lasbennes F and Gayet J: Capacity for energy metabolism in microvessels isolated from rat brain. Neurochem Res 9: 1-10, 1984.

Leeson P D and Davis A M: Time-related differences in the physical property profiles of oral drugs. J Med Chem 47: 6338-6348, 2004.

Levin V A: Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem 23: 682-684, 1980.

Li G, Simon M J, Cancel L M, Shi Z D, Ji X, Tarbell J M, Morrison B, 3rd, Fu B M: Permeability of Endothelial and Astrocyte Cocultures: In Vitro Blood-Brain Barrier Models for Drug Delivery Studies. Ann Biomed Eng, 2010.

Li J Y, Boado R J, Pardridge W M: Cloned blood-brain barrier adenosine transporter is identical to the rat concentrative Na+ nucleoside cotransporter CNT2. J Cereb Blood Flow Metab 21: 929-936, 2001.

Lipinski C A: Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods 44: 235-249, 2000.

Liu X and Chen C: Strategies to optimize brain penetration in drug discovery. Curr Opin Drug Discov Devel 8: 505-512, 2005.

Liu X, Chen C, Smith B J: Progress in brain penetration evaluation in drug discovery and development. J Pharmacol Exp Ther 325: 349-356, 2008.

Liu X, Smith B J, Chen C, Callegari E, Becker S L, Chen X, Cianfrogna J, Doran A C, Doran S D, Gibbs J P, Hosea N, Liu J, Nelson F R, Szewc M A, Van Deusen J: Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental

analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther 313: 1254-1262, 2005.

Loscher W and Potschka H: Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx 2: 86-98, 2005.

Lund-Andersen H: Transport of glucose from blood to brain. Physiol Rev 59: 305-352, 1979.

Lundquist S and Renftel M: The use of in vitro cell culture models for mechanistic studies and as permeability screens for the blood-brain barrier in the pharmaceutical industry--background and current status in the drug discovery process. Vascul Pharmacol 38: 355-364, 2002.

Maurer T S, Debartolo D B, Tess D A, Scott D O: Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. Drug Metab Dispos 33: 175-181, 2005.

McCaffrey G, Staatz W D, Quigley C A, Nametz N, Seelbach M J, Campos C R, Brooks T A, Egleton R D, Davis T P: Tight junctions contain oligomeric protein assembly critical for maintaining blood-brain barrier integrity in vivo. J Neurochem, 2007.

Meier C, Ristic Z, Klauser S, Verrey F: Activation of system L heterodimeric amino acid exchangers by intracellular substrates. Embo J 21: 580-589, 2002.

Mellman I: Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 12: 575-625, 1996.

Mueckler M: Facilitative glucose transporters. Eur J Biochem 219: 713-725, 1994.

Mueckler M and Makepeace C: Transmembrane segment 6 of the Glut1 glucose transporter is an outer helix and contains amino acid side chains essential for transport activity. J Biol Chem 283: 11550-11555, 2008.

Munn A L: Molecular requirements for the internalisation step of endocytosis: insights from yeast. Biochim Biophys Acta 1535: 236-257, 2001.

Nakagawa S, Deli M A, Nakao S, Honda M, Hayashi K, Nakaoke R, Kataoka Y, Niwa M: Pericytes from brain

microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. Cell Mol Neurobiol 27: 687-694, 2007.

Neuhaus W, Lauer R, Oelzant S, Fringeli U P, Ecker G F, Noe C R: A novel flow based hollow-fiber blood-brain barrier in vitro model with immortalised cell line PBMEC/C1-2. J Biotechnol 125: 127-141, 2006.

Nishizaki T, Kammesheidt A, Sumikawa K, Asada T, Okada Y: A sodium- and energy-dependent glucose transporter with similarities to SGLT1-2 is expressed in bovine cortical vessels. Neurosci Res 22: 13-22, 1995.

O'Kane R L, Vina J R, Simpson I, Hawkins R A: Na+ -dependent neutral amino acid transporters A, ASC, and N of the blood-brain barrier: mechanisms for neutral amino acid removal. Am J Physiol Endocrinol Metab 287: E622-629, 2004.

O'Kane R L, Vina J R, Simpson I, Zaragoza R, Mokashi A, Hawkins R A: Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y+. Am J Physiol Endocrinol Metab 291: E412-419, 2006.

Oldendorf W H, Cornford M E, Brown W J: The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. Ann Neurol 1: 409-417, 1977.

Oldendorf W H, Hyman S, Braun L, Oldendorf S Z: Blood-brain barrier: penetration of morphine, codeine, heroin, and methadone after carotid injection. Science 178: 984-986, 1972.

Olesen J, Baker M G, Freund T, di Luca M, Mendlewicz J, Ragan I, Westphal M: Consensus document on European brain research. J Neurol Neurosurg Psychiatry 77 Suppl 1: i1-49, 2006.

Omidi Y, Barar J, Ahmadian S, Heidari H R, Gumbleton M: Characterization and astrocytic modulation of system L transporters in brain microvasculature endothelial cells. Cell Biochem Funct 26: 381-391, 2008.

Pajouhesh H and Lenz G R: Medicinal chemical properties of successful central nervous system drugs. NeuroRx 2: 541-553, 2005.

Pan W, Kastin A J, Zankel T C, van Kerkhof P, Terasaki T, Bu G: Efficient transfer of receptor-associated protein (RAP) across the blood-brain barrier. J Cell Sci 117: 5071-5078, 2004.

Pardridge W M: Brain metabolism: a perspective from the blood-brain barrier. Physiol Rev 63: 1481-1535, 1983.

Pardridge W M: Blood-brain barrier transport of nutrients. Nutr Rev 44 Suppl: 15-25, 1986.

Pardridge W M: Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res 23: 635-644, 1998.

Pardridge W M: Blood-brain barrier biology and methodology. J Neurovirol 5: 556-569, 1999a.

Pardridge W M: Vector-mediated drug delivery to the brain. Adv Drug Deliv Rev 36: 299-321, 1999b.

Pardridge W M: BBB-Genomics: creating new openings for brain-drug targeting. Drug Discov Today 6: 381-383, 2001a.

Pardridge W M: Brain drug targeting, the future of brain drug development. Cambridge University Press, Cambridge 2001b.

Pardridge W M: Drug and gene delivery to the brain: the vascular route. Neuron 36: 555-558, 2002a.

Pardridge W M: Why is the global CNS pharmaceutical market so under-penetrated? Drug Discov Today 7: 5-7, 2002b.

Pardridge W M: Blood-brain barrier drug targeting: the future of brain drug development. Mol Interv 3: 90-105, 151, 2003.

Pardridge W M: Log(BB), PS products and in silico models of drug brain penetration. Drug Discov Today 9: 392-393, 2004.

Pardridge W M: The blood-brain barrier: bottleneck in brain drug development. NeuroRx 2: 3-14, 2005a.

Pardridge W M: Molecular biology of the blood-brain barrier. Mol Biotechnol 30: 57-70, 2005b.

Pardridge W M: Blood-brain barrier delivery. Drug Discov Today 12: 54-61, 2007a.

Pardridge W M: Blood-brain barrier genomics. Stroke 38: 686-690, 2007b.

Pardridge W M: Drug targeting to the brain. Pharm Res 24: 1733-1744, 2007c.

Pardridge W M and Oldendorf W H: Transport of metabolic substrates through the blood-brain barrier. J Neurochem 28: 5-12, 1977.

Pardridge W M, Triguero D, Buciak J, Yang J: Evaluation of cationized rat albumin as a potential blood-brain barrier drug transport vector. J Pharmacol Exp Ther 255: 893-899, 1990.

Park S and Sinko P J: The blood-brain barrier sodium-dependent multivitamin transporter: a molecular functional in vitro-in situ correlation. Drug Metab Dispos 33: 1547-1554, 2005.

Persidsky Y, Ramirez S H, Haorah J, Kanmogne G D: Blood-brain barrier: structural components and function under physiologic and pathologic conditions. J Neuroimmune Pharmacol 1: 223-236, 2006.

Qutub A A and Hunt C A: Glucose transport to the brain: a systems model. Brain Res Brain Res Rev 49: 595-617, 2005.

Rautio J, Laine K, Gynther M, Savolainen J: Prodrug approaches for CNS delivery. Aaps J 10: 92-102, 2008.

Regier D A, Boyd J H, Burke J D, Jr., Rae D S, Myers J K, Kramer M, Robins L N, George L K, Karno M, Locke B Z: One-month prevalence of mental disorders in the United States. Based on five Epidemiologic Catchment Area sites. Arch Gen Psychiatry 45: 977-986, 1988.

Reichel A, Begley D J, Abbott N J: Carrier-mediated delivery of metabotrophic glutamate receptor ligands to the central nervous system: structural tolerance and potential of the L-system amino acid transporter at the blood-brain barrier. J Cereb Blood Flow Metab 20: 168-174, 2000.

Rist R J, Romero I A, Chan M W, Couraud P O, Roux F, Abbott N J: F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell

monolayers: effects of cyclic AMP and astrocytic factors. Brain Res 768: 10-18, 1997.

Ritchie J W, Peter G J, Shi Y B, Taylor P M: Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in Xenopus oocytes. J Endocrinol 163: R5-9, 1999.

Rubin L L and Staddon J M: The cell biology of the blood-brain barrier. Annu Rev Neurosci 22: 11-28, 1999.

Sanchez del Pino M M, Peterson D R, Hawkins R A: Neutral amino acid transport characterization of isolated luminal and abluminal membranes of the blood-brain barrier. J Biol Chem 270: 14913-14918, 1995.

Simpson I A, Carruthers A, Vannucci S J: Supply and demand in cerebral energy metabolism: the role of nutrient transporters. J Cereb Blood Flow Metab 27: 1766-1791, 2007.

Smith Q R: Quantitation of Blood-Brain Barrier Permeability. Plenum Medical Book Company, New York 1989.

Smith Q R: Carrier-mediated transport to enhance drug delivery to brain. International Congress Series 1277: 63-74, 2005.

Smith Q R and Allen D D: In situ brain perfusion technique. Methods Mol Med 89: 209-218, 2003.

Song L, Wilk E, Wilk S, Healy D P: Localization of immunoreactive glutamyl aminopeptidase in rat brain. I. Association with cerebral microvessels. Brain Res 606: 286-294, 1993.

Stella V J, Charman W N, Naringrekar V H: Prodrugs. Do they have advantages in clinical practice? Drugs 29: 455-473, 1985.

Stewart P A, Hayakawa K, Hayakawa E, Farrell C L, Del Maestro R F: A quantitative study of blood-brain barrier permeability ultrastructure in a new rat glioma model. Acta Neuropathol 67: 96-102, 1985.

Su T Z, Lunney E, Campbell G, Oxender D L: Transport of gabapentin, a gamma-amino acid drug, by system l alpha-amino acid transporters: a comparative study in astrocytes, synaptosomes, and CHO cells. J Neurochem 64: 2125-2131, 1995.

Summerfield S G, Read K, Begley D J, Obradovic T, Hidalgo I J, Coggon S, Lewis A V, Porter R A, Jeffrey P: Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. J Pharmacol Exp Ther 322: 205-213, 2007.

Summerfield S G, Stevens A J, Cutler L, del Carmen Osuna M, Hammond B, Tang S P, Hersey A, Spalding D J, Jeffrey P: Improving the in vitro prediction of in vivo central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. J Pharmacol Exp Ther 316: 1282-1290, 2006.

Syvanen S, Xie R, Sahin S, Hammarlund-Udenaes M: Pharmacokinetic consequences of active drug efflux at the blood-brain barrier. Pharm Res 23: 705-717, 2006.

Takada Y, Vistica D T, Greig N H, Purdon D, Rapoport S I, Smith Q R: Rapid high-affinity transport of a chemotherapeutic amino acid across the blood-brain barrier. Cancer Res 52: 2191-2196, 1992.

Takasato Y, Rapoport S I, Smith Q R: An in situ brain perfusion technique to study cerebrovascular transport in the rat. Am J Physiol 247: H484-493, 1984.

Tamai I and Tsuji A: Transporter-mediated permeation of drugs across the blood-brain barrier. J Pharm Sci 89: 1371-1388, 2000.

Tao-Cheng J H and Brightman M W: Development of membrane interactions between brain endothelial cells and astrocytes in vitro. Int J Dev Neurosci 6: 25-37, 1988.

Tao-Cheng J H, Nagy Z, Brightman M W: Tight junctions of brain endothelium in vitro are enhanced by astroglia. J Neurosci 7: 3293-3299, 1987.

Thiebaut F, Tsuruo T, Hamada H, Gottesman M M, Pastan I, Willingham M C: Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. J Histochem Cytochem 37: 159-164, 1989.

Thurlow R J, Hill D R, Woodruff G N: Comparison of the uptake of [^{3}H]-gabapentin with the uptake of L-[^{3}H]-leucine into

rat brain synaptosomes. Br J Pharmacol 118: 449-456, 1996.

Tontsch U and Bauer H C: Glial cells and neurons induce blood-brain barrier related enzymes in cultured cerebral endothelial cells. Brain Res 539: 247-253, 1991.

Tsuji A: Small molecular drug transfer across the blood-brain barrier via carrier-mediated transport systems. NeuroRx 2: 54-62, 2005.

Tsuji A and Tamai I I: Carrier-mediated or specialized transport of drugs across the blood-brain barrier. Adv Drug Deliv Rev 36: 277-290, 1999.

Uchino H, Kanai Y, Kim D K, Wempe M F, Chairoungdua A, Morimoto E, Anders M W, Endou H: Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanisms of substrate recognition. Mol Pharmacol 61: 729-737, 2002.

van de Waterbeemd H, Smith D A, Jones B C: Lipophilicity in PK design: methyl, ethyl, futile. J Comput Aided Mol Des 15: 273-286, 2001.

Verrey F: System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. Pflugers Arch 445: 529-533, 2003.

Walker I N, D; Irwin W. J.; Freeman, S.: Drug delivery via active transport at the blood-brain barrier: affinity of a prodrug of phosphonoformate for the large amino acid transporter. Int. J. Pharm. 104: 157-167, 1994.

Wang Y and Welty D F: The simultaneous estimation of the influx and efflux blood-brain barrier permeabilities of gabapentin using a microdialysis-pharmacokinetic approach. Pharm Res 13: 398-403, 1996.

Watanabe K, Sawano T, Endo T, Sakata M, Sato J: Studies on intestinal absorption of sulpiride (2): transepithelial transport of sulpiride across the human intestinal cell line Caco-2. Biol Pharm Bull 25: 1345-1350, 2002.

Westergren I, Nystrom B, Hamberger A, Johansson B B: Intracerebral dialysis and the blood-brain barrier. J Neurochem 64: 229-234, 1995.

Wolburg H, Neuhaus J, Kniesel U, Krauss B, Schmid E M, Ocalan M, Farrell C, Risau W: Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes. J Cell Sci 107 (Pt 5): 1347-1357, 1994.

Wolburg H, Noell S, Mack A, Wolburg-Buchholz K, Fallier-Becker P: Brain endothelial cells and the glio-vascular complex. Cell Tissue Res 335: 75-96, 2009.

Wright E M, Hirsch J R, Loo D D, Zampighi G A: Regulation of Na+/glucose cotransporters. J Exp Biol 200: 287-293, 1997.

Yanagida O, Kanai Y, Chairoungdua A, Kim D K, Segawa H, Nii T, Cha S H, Matsuo H, Fukushima J, Fukasawa Y, Tani Y, Taketani Y, Uchino H, Kim J Y, Inatomi J, Okayasu I, Miyamoto K, Takeda E, Goya T, Endou H: Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. Biochim Biophys Acta 1514: 291-302, 2001.

3 Aims of the study

The main objective of this doctoral thesis was to develop prodrugs that are able to cross the blood-brain barrier via LAT1 or Glut1 transporters. This included the design of the prodrugs, evaluation of the physicochemical properties and evaluation of their brain uptake mechanism and the extent of the brain uptake with *in vitro*, *in situ* and *in vivo* methods. The more specific aims were:

1. To modify and combine *in vitro*, *in situ* and *in vivo* methods, in order to evaluate the mechanism of brain uptake, and the distribution in the brain parenchyma of developed amino acid and glucose prodrugs.
2. To design amino acid prodrugs of ketoprofen, which are able to bind to LAT1 and cross the blood-brain barrier.
3. To determine whether amino acids other than LAT1 substrates be used to form prodrugs that are LAT1 substrates. This would enable the use of different prodrug bonds between the parent drug and the amino acid. L-Lysine was chosen as a promoiety, because it is not a LAT1 substrate and it can be conjugated with ketoprofen via the amide bond on its side chain.
4. To determine the ability of the amino acid prodrugs to deliver ketoprofen into the brain parenchyma *in vivo*.
5. To design D-glucose prodrugs of ketoprofen and indomethacin which are able to bind to GluT1 and cross the blood-brain barrier.

4 Experimental

4.1. ANALYTICAL METHODS

4.1.1 HPLC assay

The prodrug and parent drug concentrations were analyzed by Agilent 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Karlsruhe, Germany) that consisted of a binary pump G1312A, a vacuum degasser G1379A, an automated injector system autosampler Hewlett Packard 1050, an UV-detector Hewlett Packard 1050 variable wavelength detector and an analyst software Agilent ChemStation for LC Systems Rev. A.10.02. The detector wavelength was set at 256 nm when ketoprofen or ketoprofen prodrug were analyzed and at 222 nm when indomethacin prodrugs were analyzed. A mixture of acetonitrile and a 0.02 M phosphate buffer solution at a flow rate of 1 mL/min was used as a mobile phase. Reversed-phase HPLC was conducted with a Zorbax RP-18 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Little Falls, Wilmington, DE).

4.1.2 Liquid chromatography-electrospray tandem mass spectrometry (LC-MS)

The quantitative determination of ketoprofen-lysine amide and ketoprofen in plasma and brain samples was performed using liquid chromatography-electrospray tandem mass spectrometry (LC-MS) (Agilent 1200 Series Rapid Resolution LC System, Agilent Technologies, Waldbronn, Germany) coupled with an electrospray ionization (ESI) triple quadrupole mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS, Agilent Technologies, Palo Alto, CA). The chromatographic separation was performed on a reversed phase column by gradient method with acetonitrile and water, both containing 1% formic acid at a

flow-rate of 0.3 mL/min. Positive electrospray ionization and selected reaction monitoring were used for the detection of ketoprofen-lysine amide (*m*/*z* 383 → 84) and ketoprofen (*m*/*z* 255 → 209).

4.1.3 Brain and plasma sample preparation

L-Tyrosine-ketoprofen, L-lysine-ketoprofen, D-glucose-ketoprofen and D-glucose-indomethacin prodrugs and ketoprofen were isolated from the rat brain homogenate samples by liquid-liquid extraction. A complete brain hemisphere was homogenated with 2.5 mL of water to produce 3.0 mL of homogenate. The samples were acidified with 300 µl of 2 M hydrochloric acid and vortexed for 5 min. Solvent was added and aliquots were vortexed for 2 min, and centrifuged for 10 min (7500 × g) after which the supernatants were collected. The supernatants were applied to the preconditioned and equilibrated C18 solid phase extraction cartridges. The extracted supernatants were evaporated to dryness under a nitrogen stream at 40 °C. Prior to LC-MS or HPLC-UV analysis, the samples were reconstituted in 400 µL acetonitrile-water solution, and filtered.

L-Lysine-ketoprofen prodrug and ketoprofen and were isolated from the plasma samples by protein precipitation. Plasma (100 µL) was acidified with 50 µL of 1 M hydrochloric acid and vortexed for 5 min. Acetonitrile (1.0 mL) was added and the samples were vortexed for 2 min, after which time the samples were centrifuged for 5 min (5500 × g). The supernatants were collected, and the samples were evaporated to dryness under a nitrogen stream at 40 °C. Prior to LC-MS or HPLC-UV analysis, samples were reconstituted in 400 µL acetonitrile-water solution, and filtered.

4.2 PHYSICOCHEMICAL PROPERTIES OF THE PRODRUGS

4.2.1 Apparent partition coefficients

The apparent partition coefficients (LogD) were determined at room temperature by a 1-octanol-phosphate buffer system at pH 7.4. Before use, the 1-octanol was saturated with phosphate buffer for 24 h by stirring vigorously. A known concentration of compound in phosphate buffer was shaken 30 min (pH 7.4), with a suitable volume of 1-octanol. After shaking, the phases were separated by centrifugation at 14000 rpm for 4 min. The concentrations of the compounds in the buffer phase before and after partitioning were determined by HPLC.

4.2.2 Polar surface area

Polar surface areas of prodrugs were calculated using the method by Ertl et al. (Ertl et al., 2000) implemented in Molecular Operating Environment.

4.2.3 *In vitro* brain tissue and plasma protein binding

Drug-naive animals were sacrificed, the brain was removed, and 3 volumes of a phosphate buffered saline (pH 7.4) was added. The brains were homogenized on ice with an ultrasonic probe after which the drug was added. Blood was recovered from anesthetized animal with heart puncture and plasma was separated by centrifugation. Equilibrium dialysis of 400 μL of homogenate or plasma and 600 μL of buffer was performed in triplicate for 4 h at 37 °C in Single-Use RED Plate with an 8-kDa cutoff dialysis membrane (Thermo Scientific, Rockford, IL). The buffer to homogenate or plasma concentration ratio was calculated, which was used to calculate the *in vitro* brain tissue and plasma protein binding (Kalvass and Maurer, 2002; Friden et al., 2007).

4.2.4 CHEMICAL AND ENZYMATIC STABILITY OF THE PRODRUGS *IN VITRO*

The rate of chemical hydrolysis of the prodrugs was studied in aqueous phosphate buffer solution of pH 7.4 (0.16 M, ionic strength 0.5) at 37 °C. An appropriate amount of the prodrugs was dissolved in 10 mL of preheated buffer and the solutions were placed in a thermostatically controlled water bath at 37 °C. At appropriate intervals, samples were taken and analyzed for remaining prodrug by the HPLC. Pseudo-first-order half-time ($t_{1/2}$) for the hydrolysis of the prodrugs was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

The rate of enzymatic hydrolysis of the prodrugs was studied at 37 °C in 80% (v/v) rat serum, in 20% rat brain homogenate and in 50% rat liver homogenate. The concentration of esterase enzymes in the homogenates was not determined. The mixtures were kept in a water bath at 37 °C, and 0.2 mL of serum/buffer or homogenate/buffer mixture was withdrawn and added to 0.2 mL of acetonitrile to precipitate protein from the sample. After immediate mixing and centrifugation, the supernatant was analyzed for the remaining prodrug and released the parent drug by the HPLC. Pseudo-first-order half-time ($t_{1/2}$) for the hydrolysis of the prodrugs was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time.

4.3 ANIMALS

Adult male Wistar rats (250 g) were supplied by the National Laboratory Animal Centre (Kuopio, Finland) for the *in vivo* studies. Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/mL, i.p.).

4.4 *IN SITU* RAT BRAIN PERFUSION TECHNIQUE

In the present study, the *in situ* rat brain perfusion technique was used for the determination of the brain uptake mechanism of the prodrugs, since it has advantages over both *in vivo* and *in vitro* techniques. The experimental conditions can be easily manipulated to study saturable processes such as carrier-mediated uptake, and moreover, experimental conditions can be used that would be toxic in the *in vivo* situation. The most important advantage over *in vivo* experiments is the simplicity of the pharmacokinetics that makes the accurate determination of the uptake mechanism of the prodrug possible (Foster and Roberts, 2000; Summerfield et al., 2007). *In vivo* techniques take into account not only BBB penetration but also binding to plasma proteins, metabolism, and clearance and there is significant value in removing some of this complexity and assessing brain penetration at the level of the BBB *in situ*. Furthermore, the *in situ* technique provides deeper insight into the molecular descriptors that are crucial for BBB penetration (Summerfield et al., 2007). The most important advantage over in vitro techniques is that the BBB is in its normal physiological state when *in situ* technique is used.

Modified *in situ* rat brain perfusion technique (Smith and Allen, 2003; Killian et al., 2007) was used to quantify the brain uptake and the transport mechanism of the prodrugs at the BBB. The right carotid artery of the rats was exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with PE-50 catheters filled with 100 IE/mL heparin. The right occipital and the right pterygopalatine arteries were left open. The blood flow of the rats was interrupted by severing the cardiac ventricles. The perfusion fluid was infused through the common carotid artery at the rate of 10 mL/min for 30–60 seconds using Harvard PHD 22/2000 syringe pump (Harvard apparatus Inc., Holliston, MA). After decapitation, the skull was opened and brain sections (10–30 mg) were excised from six different regions of the right brain hemisphere (frontal cortex, parietal cortex, occipital cortex,

hippocampus, caudate nucleus and thalamus). Left parietal cortex was used as the control. All samples were analyzed for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland). Brain samples were dissolved in 0.5 mL of Solvable™ (PerkinElmer, Boston, MA) overnight at 50 °C and liquid scintillation cocktail (Ultima Gold, PerkinElmer, Boston, MA) was added before analyzing the samples. The perfusion medium consisted of a pH 7.4 bicarbonate-buffered physiological saline (128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 9 mM D-glucose). The solution was filtered, heated to 37 °C and bubbled with 95% O_2 / 5% CO_2 to attain steady state gas levels within the solution. The *in situ* rat brain technique was validated by measuring the intravascular volume (V_v) as estimated by [^{14}C]sucrose and by quantifying the brain uptake of [^{3}H]diazepam, [^{14}C]urea and [^{14}C]L-leucine. All radiolabeled compounds used in the *in situ* rat brain perfusion technique were purchased from PerkinElmer (Boston, MA) and were uniformly labeled.

4.5 DETERMINATION OF THE BRAIN UPTAKE MECHANISM FOR PRODRUGS

The ability of prodrugs to bind to LAT1 or GluT1 was studied with the *in situ* rat brain perfusion technique. The 100% PA products of [^{14}C]L-leucine and [^{14}C]D-glucose were determined after 30 s perfusion of 0.2 μCi/mL [^{14}C]L-leucine or [^{14}C]D-glucose solution. In the competition studies, the radiolabeled substrates were co-perfused with prodrugs for 30 s. In order to study whether the binding of the prodrugs to the transporter was reversible, the PA product of radiolabeled substrate was determined after perfusing rat brain first with the prodrug for 30 s, followed by washing the prodrug from the brain capillaries with prodrug-free perfusion medium for 30 s and finally perfusing the brain with radiolabeled substrate for 30 s.

4.6 BRAIN UPTAKE STUDIES OF THE PRODRUGS

The brain uptake studies of the L-tyrosine-ketoprofen, L-lysine-ketoprofen, D-glucose-ketoprofen and D-glucose-indomethacin prodrugs were performed with the *in situ* rat brain perfusion technique. The prodrugs were dissolved in dimethyl sulfoxide (DMSO) and then added to the perfusion medium resulting in 1% (v/v) DMSO solution. After adjusting the pH to 7.4, the solution was filtered with 0.45 μm Millex-HV filters. The rat brains were perfused 60 s with 37 °C perfusion medium containing the prodrug. After perfusion, the remaining prodrug was washed from the brain vasculature with cold prodrug-free perfusion medium (5 °C) for 30 s. The prodrug concentration of the perfusion medium was analyzed by the HPLC after each perfusion to confirm that the prodrug had stayed intact. The brain uptake was also determined by inhibiting carrier-mediated uptake non-specifically at a low temperature. The brain capillaries were first washed for 30 s with cold prodrug-free perfusion medium (5 °C), followed by perfusion for 60 s with cold prodrug solution (5 °C), and washed again with cold prodrug-free perfusion medium (5 °C).

4.7 CAPILLARY DEPLETION ANALYSIS

Capillary depletion analysis was carried out as previously described by (Triguero et al., 1990). Brain samples (right brain hemisphere) were weighed and homogenized in glass homogenizer with 1.5 mL of physiological buffer. After homogenization, 2 mL of 26% dextran solution was added and the mixture was further homogenized. The homogenate was separated into two microcentrifuge tubes and centrifuged 15 minutes (5400 × g, 4 °C). The resulting supernatant consisting of the brain parenchyma and the pellet rich in cerebral capillaries were separated and prepared for analysis with the HPLC. Values of the volume of distribution (V_d) for the homogenate, supernatant fraction and the capillary pellet were calculated.

4.8 *IN VIVO* INTRAVENOUS BOLUS INJECTION METHOD

L-Lysine-ketoprofen prodrug and ketoprofen were dissolved in 0.9% NaCl solution of pH 7.4 resulting in 30 mM concentration. Then, a 0.2 mL bolus injection of either drug was administered into the cannulated jugular vein of the rats, and plasma and brain concentrations were determined at the following time points: 10, 30, 60, 120 and 300 min. For V_d and clearance (C_L) determinations the plasma samples were taken in following time points: 2, 5, 10, 15, 30, 45, 60, 90 and 120 min. The plasma samples were drawn from the cannulated jugular vein at different time points and the brain were removed after the rats were sacrificed. Ketoprofen and prodrug levels in brain parenchyma were calculated by correcting the measured brain sample concentration for the cerebral vascular space component, which was determined to be 0.0116 mL/g.

4.9 *IN VIVO* MICRODIALYSIS

A microdialysis guide cannula (MAB 6.10.IC, AgnTho's AB, Lidingö, Sweden) was implanted into the striatum (coordinates from bregma: AP +0.5 mm; L -3.0 mm; DV -3.8 mm) under chloral hydrate anesthesia (350 mg/kg i.p.). After a one week recovery period, the rats were again anesthetized and a concentric microdialysis probe (MAB 9.10.4; 4 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the striatum through the guide cannula. An intravenous microdialysis probe (MAB 11.20.10; 10 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the left femoral vein, and both probes were perfused with Krebs Ringer solution (consisting of 138 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2 \bullet 6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \bullet H_2O$, and 11 mM D-glucose, pH 7.4) at a flow rate of 2 μl/min. For drug administration, the right femoral vein was cannulated with polyethylene tubing filled with saline and 20 IU/ml dalteparin. The microdialysis probes were perfused for 80 min before

intravenous drug administration, and the dialysate was collected as ten or twenty-minute fractions for 5 hours into polypropylene vials (AgnTho's AB). All dialysate drug concentrations were corrected with recovery values. The samples were frozen at -20 °C and stored at -80 °C until analyzed.

4.10 *IN VITRO* RECOVERY OF MICRODIALYSIS PROBES

To estimate the true free drug concentration in brain ECF and the blood probe calibration was performed *in vitro* for both probe types. The recovery was determined as the ratio of a drug in the dialysate to the drug concentration (1 μM) in a non-stirred bulk Krebs Ringer solution at 37 °C (C_{dial}/C_{bulk}; n=3 probes for each drug, 3 determinations per probe; flow rate 2 μl/min, collection time 20 min).

4.11 EQUATIONS

All used equations are presented in chapter 2.4.1.

4.12 REFERENCES

Ertl P, Rohde B, Selzer P: Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J Med Chem 43: 3714-3717, 2000.

Foster K A and Roberts M S: Experimental methods for studying drug uptake in the head and brain. Curr Drug Metab 1: 333-356, 2000.

Friden M, Gupta A, Antonsson M, Bredberg U, Hammarlund-Udenaes M: In vitro methods for estimating unbound drug concentrations in the brain interstitial and intracellular fluids. Drug Metab Dispos 35: 1711-1719, 2007.

Kalvass J C and Maurer T S: Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. Biopharm Drug Dispos 23: 327-338, 2002.

Killian D M, Hermeling S, Chikhale P J: Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. Drug Deliv 14: 25-31, 2007.

Smith Q R and Allen D D: In situ brain perfusion technique. Methods Mol Med 89: 209-218, 2003.

Summerfield S G, Read K, Begley D J, Obradovic T, Hidalgo I J, Coggon S, Lewis A V, Porter R A, Jeffrey P: Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. J Pharmacol Exp Ther 322: 205-213, 2007.

Triguero D, Buciak J, Pardridge W M: Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. J Neurochem 54: 1882-1888, 1990.

5 LAT1 - Mediated brain uptake of amino acid prodrugs of ketoprofen

Abstract. LAT1 is expressed on the luminal and abluminal membrane of the BBB capillary endothelial cells and it efficiently transports large neutral L-amino acids into the brain. The efficient brain uptake of amino acids as well as some CNS drugs via LAT1 suggests that the transporter could also be used to target prodrugs into the brain. We describe a feasible means to achieve carrier-mediated drug transport into the rat brain via LAT1 by conjugating a model compound to amino acids. A hydrophilic drug, ketoprofen, that is not a substrate for LAT1 was chosen as a model compound. Out of five amino acid prodrugs of ketoprofen that were designed, synthesized and evaluated, one prodrug L-tyrosine-ketoprofen ester showed affinity for LAT1. The mechanism and the kinetics of the brain uptake of the prodrug were determined with *in situ* rat brain perfusion technique. The brain uptake of the prodrug was found to be concentration dependent. In addition, LAT1 inhibitor significantly decreased the brain uptake of the prodrug. Therefore, our results show that the amino acid prodrug approach can achieve brain uptake of small molecular weight drugs *in situ* via LAT1.

Adapted with permission of the American Chemical Society from: Gynther M., Laine K., Ropponen J., Leppänen J., Mannila A., Nevalainen T., Savolainen J., Järvinen T., RautioJ.: Large neutral amino acid transporter enables brain drug delivery via prodrugs. Journal of Medicinal Chemistry 51: 932-936, 2008.

5.1 INTRODUCTION

Brain penetration is essential for drugs intended to act within the CNS. The entry of drug molecules into the CNS is efficiently governed by the BBB (Pardridge, 2003; Begley, 2004). Many pharmacologically active drugs intended for CNS disorders fail early in their development phase because they lack the structural features essential for crossing the BBB and distributing into the brain parenchyma. Therefore, CNS drugs should be designed with appropriate brain penetration properties (Liu et al., 2008). Good BBB penetration can be achieved by designing highly permeable compounds and by screening out efflux transporter substrates (Liu et al., 2008). One attractive approach to achieve good BBB penetration and low brain non-specific tissue binding, without losing the pharmacological activity, is to utilize the prodrug approach (Rautio et al., 2008). The properties of a drug molecule can be altered in a bio-reversible manner by conjugating the drug with a suitable promoiety.

Several specific endogenous influx transporters *e.g.* LAT1 have been identified on the brain capillary endothelial cells which form the BBB. LAT1 is expressed on the luminal and abluminal membranes of the capillary endothelial cells and it efficiently transports neutral L-amino acids (*e.g.*, phenylalanine and leucine) into the brain. If one could achieve selective release of the active drug in the target tissue, this would result in a reduced plasma concentration of the drug and fewer peripheral effects.

The aim of the study was to design and synthesize amino acid prodrugs of ketoprofen and to evaluate their ability to cross the BBB via LAT1. The mechanism of brain uptake of the prodrugs was determined with *in situ* rat brain perfusion technique.

5.2 RESULTS AND DISCUSSION

5.2.1 Design of the prodrugs

Ketoprofen was chosen as a model compound because it is a fairly stable molecule in various mediums, it is easy to detect with UV-detector or mass spectrometry and it has a carboxyl group, which makes it easy to conjugate with various promoieties. In addition, it has been proposed that non-steroidal anti-inflammatory drugs may have some therapeutic effects in CNS disorders, such as Alzheimer`s disease.

The prodrugs were designed to meet the requirements for LAT1 substrates based on the proposed LAT1 binding site model (Uchino et al., 2002; Smith, 2005). That model states that a potential LAT1 substrate should have a positively charged amino group, a negatively charged carboxyl group and a hydrophobic side chain. L-Tyrosine was selected because the phenolic hydroxyl group enables the formation of ester bond between ketoprofen and the amino acid (Fig. 5.1) L-Phenylalanine and L-leucine prodrugs were synthesized in order to confirm that free carboxylic and amino groups are indeed required for LAT1 recognition. L-Phenylalanine and L-leucine do not have suitable functional groups in their side chains for prodrug bond. Therefore, ketoprofen has to be conjugated with the amino acids either from the carboxylic or the amino groups.

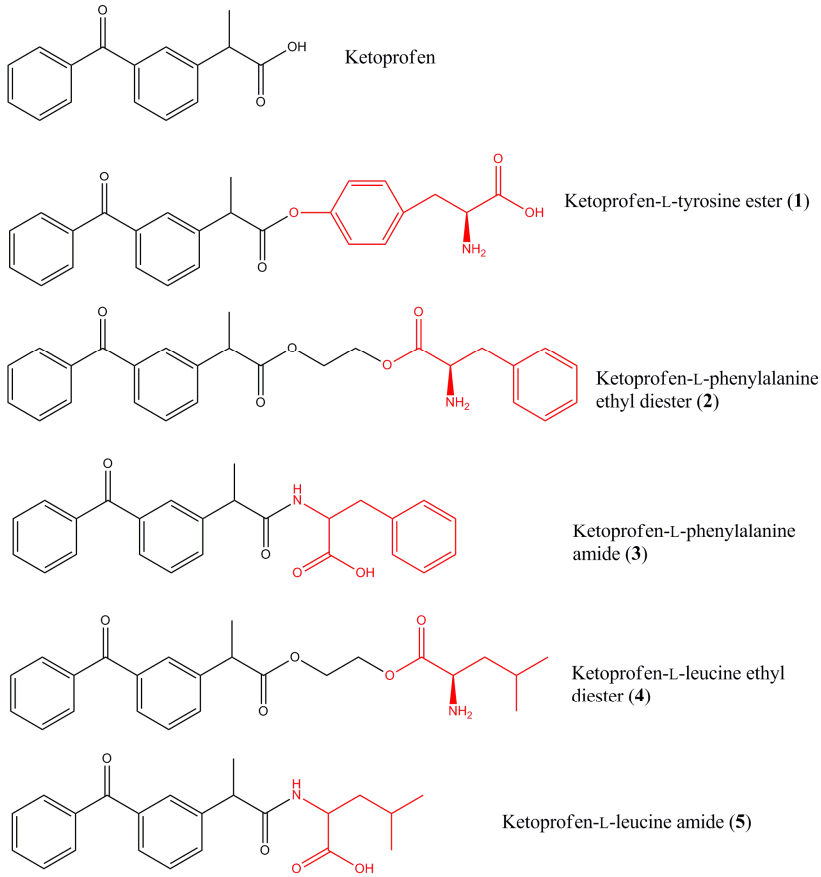


Figure 5.1. Chemical structures of ketoprofen and the amino acid prodrugs **1**-**5**.

5.2.2 Chemical and enzymatic stability of the prodrugs

The degradation of the prodrugs was studied in aqueous buffer solution of pH 7.4 at 37 °C. The degradation of L-tyrosine-ketoprofen prodrug (**1**) followed pseudo-first-order kinetics with a half-life of 22.5 ± 1.4 hours (mean ± s.d., n=2). Therefore, the prodrug demonstrated sufficient chemical stability in aqueous solutions for further evaluation.

If the target for a prodrug design is the site-selective drug delivery to the CNS, the prodrug must not only be delivered to its intended site of action, but its bioconversion should also be selective (Anderson, 1996). **1** was highly susceptible to enzymatic hydrolysis as it was quantitatively cleaved to

ketoprofen and L-tyrosine in 80% rat serum, in 20% rat brain homogenate and in 50% rat liver homogenate. The enzymatic hydrolysis followed pseudo-first-order kinetics, the half-lives being 10.2 ± 0.4, 4.4 ± 1.8 and 2.8 ± 0.6 minutes (mean ± s.d., n=3) in serum, brain homogenate and liver homogenate, respectively. Therefore, **1** undergoes rapid bioconversion to ketoprofen and L-tyrosine in the brain tissue. However, **1** is also highly susceptible to enzymatic hydrolysis in both rat serum and liver which may compromise its effective brain drug delivery.

5.2.3 *In situ* rat brain perfusion technique

In situ rat brain perfusion technique was used to evaluate intravascular volume (V_v) of the rat brain, the cerebral perfusion flow rate (F) and the brain capillary permeability-surface area (PA) product of [^{14}C]L-leucine and [^{14}C]urea. The presence of functional LAT1-transporters was evaluated based on recent publications (Rousselle et al., 1998; Killian and Chikhale, 2001; Smith and Allen, 2003; Killian et al., 2007). 0.2 μCi/mL [^{14}C]sucrose was used as a vascular marker to determine V_v and to demonstrate the integrity of the BBB during brain perfusion (Killian et al., 2007). The value of V_v was determined to be 0.0116 ± 0.0013 mL/g (mean ± s.d., n=3). The integrity of the brain capillaries was tested with 0.2 μCi/mL [^{14}C]sucrose after addition of 1% (v/v) of dimethyl sulfoxide (DMSO). The value of V_v was 0.01032 ± 0.00098 mL/g (mean ± s.d., n=3) indicating that the addition of 1% (v/v) of DMSO does not compromise the integrity of the tight junctions between the endothelial cells.

A highly diffusible and lipophilic solute, [^{3}H]diazepam, was used to determine F under the test conditions (Rousselle et al., 1998). The value of F for 0.2 μCi/mL [^{3}H]diazepam was determined to be 0.02846 ± 0.00395 mL/s/g (mean ± s.d., n=4). The value of F for [^{3}H]diazepam was also determined using 5 °C perfusion medium and it was 0.02561 ± 0.0045 mL/s/g (mean ± s.d., n=2). Similar F values indicate that the low temperature of the perfusion medium did not have any effect on the passive diffusion of [^{3}H]diazepam across the BBB.

The permeability of rat BBB in the test system was studied with [^{14}C]urea, a polar molecule with low BBB permeability (Rousselle et al., 1998; Killian and Chikhale, 2001). PA product of 0.2 μCi/mL [^{14}C]urea was determined to be 0.00018 ± 0.000056 mL/s/g (mean ± s.d., n=3). The low permeability of urea was in agreement with previous studies (Rousselle et al., 1998; Killian and Chikhale, 2001).

The presence of functional LAT1-transporters in rat BBB was confirmed with L-leucine, which is an endogenous substrate for the LAT1 (Duelli et al., 2000). The PA product of 0.2 μCi/mL [^{14}C]L-leucine was determined to be 0.02059 ± 0.00323 mL/s/g (mean ± s.d., n=5). The PA product was also determined using 5 °C perfusion medium with PA product of 0.0037 ± 0.00023 mL/s/g (mean ± s.d., n=3), suggesting that the uptake of L-leucine in the *in situ* rat brain perfusion test method was carrier-mediated, since the carrier-mediated uptake is reduced when the temperature is lowered (Kageyama et al., 2000). In addition, the determination of cerebrovascular LAT1 functional expression was carried out with competition assay by perfusing [^{14}C]L-leucine (0.2 μCi/mL) with 2 mM concentration of another known LAT1 substrate, L-phenylalanine (Killian and Chikhale, 2001). This co-perfusion resulted in almost complete inhibition (99%) of [^{14}C]L-leucine brain uptake, thereby demonstrating functional expression of cerebrovascular LAT1, which is in agreement with the literature (Killian and Chikhale, 2001).

5.2.4 Determination of the brain uptake mechanism for prodrugs

The ability of the prodrugs to bind into LAT1 was studied with the *in situ* rat brain perfusion technique. In the presence of 70 μM L-tyrosine-ketoprofen prodrug or 90 μM L-lysine-ketoprofen prodrug, the [^{14}C]L-leucine PA product was significantly decreased from 0.02059 ± 0.00323 mL/s/g (mean ± s.d., n=5) to 0.000317 ± 0.000136 mL/s/g (mean ± s.d., n=3) (98.5% inhibition) and 0.00426 ± 0.00019 mL/s/g (mean ± s.d., n=3) (79.3% inhibition), respectively. This is a clear evidence of significant

binding of the prodrugs to the LAT1 (Fig. 5.2). To further study the binding kinetics of the prodrugs to LAT1, the PA product of [^{14}C]L-leucine was determined after perfusing rat brain first with the prodrugs for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium and finally perfusing the rat brain with 0.2 μCi/mL [^{14}C]L-leucine for 30 s. The PA product of [^{14}C]L-leucine had recovered, indicating that the binding of L-tyrosine-ketoprofen prodrug and L-lysine-ketoprofen prodrug to the LAT1 is reversible (Fig. 5.2).

To support the proposed structural requirements for LAT1 substrates reported by (Uchino et al., 2002) we synthesized two ketoprofen prodrugs with phenylalanine promoiety (**2** and **3**) and two prodrugs with leucine promoiety (**4** and **5**). Ketoprofen was conjugated with the carboxyl group by diethylester linker or the amino group to remove the zwitter ionic properties of the amino acids that are necessary for LAT1 recognition. The 100% PA product of [^{14}C]L-leucine was determined after 30 s perfusion of 0.2 μCi/mL [^{14}C]L-leucine solution. In a competition study [^{14}C]L-leucine (0.2 μCi/mL) was co-perfused with 70 μM concentration of the prodrugs for 30 s. In the presence of phenylalanine and leucine prodrugs, the PA product of [^{14}C]L-leucine was 0.02815 ± 0.0059 (**2**), 0.01910 ± 0.0063 (**3**), 0.02117 ± 0.0072 (**4**), 0.02655 ± 0.0018 (**5**) (mean ± s.d., n=2). This indicates that none of these four prodrugs was able to bind to LAT1, which is in correlation with the literature (Uchino et al., 2002). Therefore, our results support the previous reports that the potential substrate should have positively charged amino group, a negatively charged carboxyl group and a hydrophobic side chain. As none of the phenylalanine or leucine prodrugs was able to bind to LAT1, these prodrugs were not studied further.

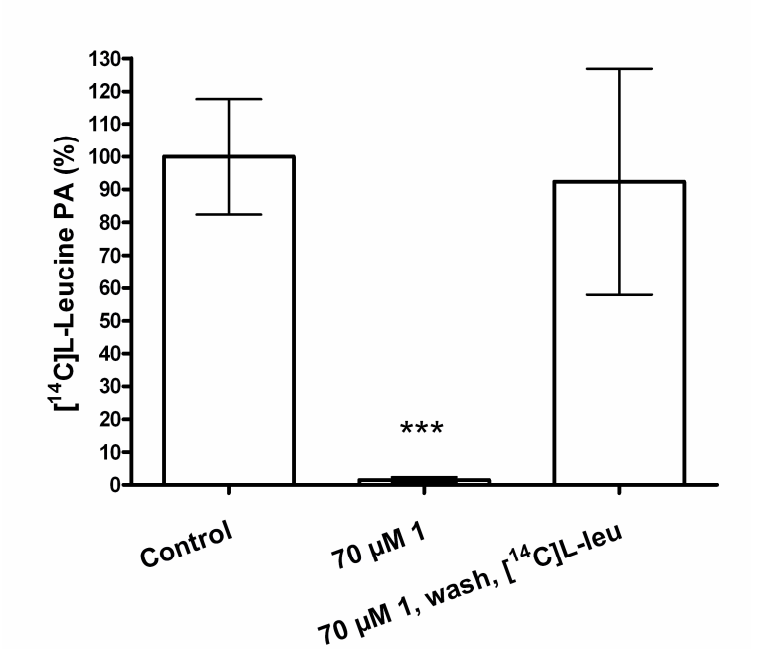


Figure 5.2. Mechanism of rat brain uptake of the prodrugs. The PA product of 0.2 μCi/mL [^{14}C]L-leucine in absence or presence of **1**. The control PA product 0.02059 ± 0.00323 mL/s/g (mean ± s.d., n=5) is decreased to 0.000317 ± 0.000136 mL/s/g in the presence of 70 μM **1** (98.5% inhibition). The uptake of [^{14}C]L-leucine is recovered after washing the prodrug from the brain capillaries demonstrating the PA product of 0.01902 ± 0.0058 mL/s/g. The data is presented as mean ± s.d. (n=3). An asterisk denotes a statistically significant difference from the respective control (***P<0.001, followed by Dunnett t-test).

After demonstrating affinity for LAT1, the *in situ* rat brain uptake of the prodrug was determined to be concentration dependent in the dose range from 0 μM to 138 μM for **1** (Fig. 5.3), indicative of transporter-mediated uptake. In addition, the brain uptake of 64 μM **1** was 0.846 ± 0.069 pmol/mg/min and addition of 2 mM 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid (BCH), a specific LAT1 substrate (Kageyama et al., 2000), to the perfusion medium significantly decreased the brain uptake

to 0.362 ± 0.065 pmol/mg/min (Fig. 5.4), evidence that the uptake was LAT1-mediated (Fig. 5.4).

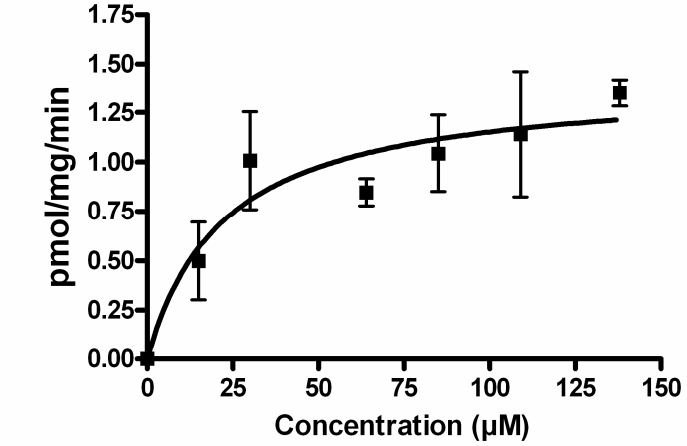


Figure 5.3. Kinetics of the rat brain uptake of the prodrugs. K_m and V_{max} are 22.49 ± 9.18 μM and 1.41 ± 0.15 pmol/mg/min (mean ± s.d., n=3) for **1**.

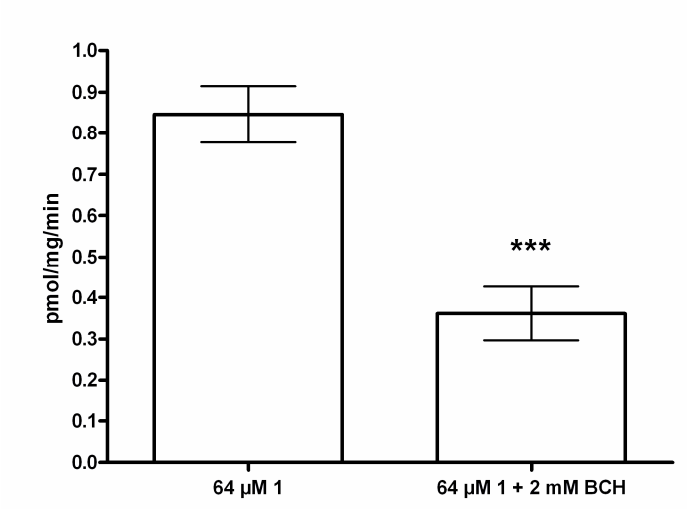


Figure 5.4. The brain uptake of 64 μM **1**. The brain uptake of **1** decreased from 0.846 ± 0.069 pmol/mg/min to 0.362 ± 0.065 pmol/mg/min in the presence of 2 mM BCH. The data is

presented as mean ± s.d. (n=3). The decrease of the uptake is statistically significant (***P=0.001, t-test).

5.2.5 Capillary depletion analysis

Capillary depletion analysis of brain samples from perfused brain showed that **1** is present in the supernatant fraction which consists of brain parenchyma (Table 5.1). The concentration of **1** in the endothelial cell enriched pellet fraction was below the lower limit of detection. However, since the uptake of **1** determined from the whole brain is higher compared to brain parenchyma, a fraction of **1** is probably captured into the endothelial cells.

Table 5.1. Capillary depletion analysis after 60 s perfusion of **1** prodrug with 85 μM concentration, followed by washing the prodrug from the capillaries with 30 s perfusion.

Right cerebrum	V_d (mL/g)
Whole brain	0.0123 ± 0.0016
Supernatant	0.0118 ± 0.0008
Pellet	-[a]

mean ± s.d., n=3, a below lower limit of detection

5.2.6 Data Analyses

Statistical differences between groups were tested using one-way ANOVA, followed by two-tailed Dunnett t-test (Fig. 5.2). In figure 5.3, two tailed independent samples t-test was used. $P<0.05$ was considered as statistically significant. Data analyses for the dose-uptake curve (Fig. 5.4) were calculated as nonlinear regressions using GraphPad Prism 4.0 for Windows. The normality of the data was tested using Shapiro-Wilk test. All statistical analyses were performed using SPSS 14.0 for Windows.

5.3 CONCLUSIONS

In the present study we were able to demonstrate that amino acid prodrugs of ketoprofen are able to cross the BBB LAT1-mediatedly. In addition, our results supported the earlier proposal that the parent drug has to be conjugated with the amino acid promoiety from the side chain in order to maintain the affinity for LAT1. One of the synthesized prodrugs, **1**, was able to utilize LAT1 for brain permeation. Our results show that the prodrug is able to significantly inhibit the LAT1-mediated uptake of L-leucine, the brain uptake of the prodrug was saturable and the uptake was inhibited by LAT1 substrate.
Although **1** was too labile to be tested *in vivo* and the present study evaluated only the brain uptake of this ketoprofen prodrug *in situ*, the strategy may offer a potential way to achieve shuttling of other small molecular weight CNS drugs especially with low BBB permeation, into the brain.

5.4 REFERENCES

Anderson B D: Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19: 171-202, 1996.

Begley D J: Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 104: 29-45, 2004.

Duelli R, Enerson B E, Gerhart D Z, Drewes L R: Expression of large amino acid transporter LAT1 in rat brain endothelium. J Cereb Blood Flow Metab 20: 1557-1562, 2000.

Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N, Shimohama S: The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. Brain Res 879: 115-121, 2000.

Killian D M and Chikhale P J: Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature. Neurosci Lett 306: 1-4, 2001.

Killian D M, Hermeling S, Chikhale P J: Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. Drug Deliv 14: 25-31, 2007.

Liu X, Chen C, Smith B J: Progress in brain penetration evaluation in drug discovery and development. J Pharmacol Exp Ther 325: 349-356, 2008.

Pardridge W M: Blood-brain barrier drug targeting: the future of brain drug development. Mol Interv 3: 90-105, 151, 2003.

Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, Savolainen J: Prodrugs: design and clinical applications. Nat Rev Drug Discov 7: 255-270, 2008.

Rousselle C H, Lefauconnier J M, Allen D D: Evaluation of anesthetic effects on parameters for the in situ rat brain perfusion technique. Neurosci Lett 257: 139-142, 1998.

Smith Q R: Carrier-mediated transport to enhance drug delivery to brain. International Congress Series 1277: 63-74, 2005.

Smith Q R and Allen D D: In situ brain perfusion technique. Methods Mol Med 89: 209-218, 2003.

Uchino H, Kanai Y, Kim D K, Wempe M F, Chairoungdua A, Morimoto E, Anders M W, Endou H: Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanisms of substrate recognition. Mol Pharmacol 61: 729-737, 2002.

6 Brain uptake and intracerebral distribution of ketoprofen-amino acid prodrugs

Abstract. The efficient brain uptake of amino acids as well as some CNS drugs via LAT1 suggests that the transporter could also be used to target prodrugs into the brain. A hydrophilic drug, ketoprofen, that is not a substrate for LAT1 was chosen as a model compound. L-lysine-ketoprofen amide showed affinity for LAT1. The mechanism and the kinetics of the brain uptake of the prodrug were determined with *in situ* rat brain perfusion technique. The brain uptake of the prodrug was found to be concentration dependent. In addition, LAT1 inhibitor significantly decreased the brain uptake of the prodrug. Furthermore, the ability to deliver ketoprofen into the brain ICF *in vivo* was evaluated for L-lysine-ketoprofen amide. The L-lysine prodrug was able to deliver ketoprofen into the intracellular compartment of brain cells. Therefore, our results show that the amino acid prodrug approach can achieve brain uptake of small molecular weight drugs *in vivo* via LAT1.

Adapted with of permission Elsevier B.V. from: Gynther M., Laine K., Ropponen J., Leppänen J., Lehtonen M., Kauvosaari J., Jalkanen A., Forsberg M., Rautio J.: Brain uptake of ketoprofen-lysine prodrug in rats. International Journal of Pharmaceutics 399: 121-128, 2010.

6.1 INTRODUCTION

BBB permeability and the non-specific interaction with brain tissue are important factors for uptake optimization (Summerfield et al., 2006). In cases where the site of action is inside the cell, the cellular uptake of drugs is as important as the BBB permeability (de Lange and Danhof, 2002). One attractive approach to achieve good BBB penetration and low brain non-specific tissue binding, without losing the pharmacological activity, is to utilize the prodrug approach (Rautio et al., 2008). The properties of a drug molecule can be altered in a bio-reversible manner by conjugating the drug with a suitable promoiety. Traditionally, attempts have been made to overcome the poor brain uptake of CNS drugs by increasing the lipophilicity of the drug with a lipophilic promoiety. Increasing the lipophilicity of a poorly permeable drug molecule often leads to an increase in BBB permeability (Summerfield et al., 2006; Summerfield et al., 2007). However, at same time, the increased lipophilicity may increase accumulation of the drug in peripheral tissues. Therefore, the percentage of the given dose that gains the access into the brain *in vivo* may actually be decreased. In addition, the binding to plasma protein is often increased due to increased lipophilicity, which may decrease the brain uptake. On the other hand, the lowering of the lipophilicity of highly tissue bound drugs often results in poor BBB permeability. Due to the complex role of the drug lipophilicity in brain penetration, the traditional prodrug approach, which deals with the hydrophilicity and lipophilicity of the drugs, is often not suitable and instead, a more sophisticated prodrug approach is needed.

Several specific endogenous influx transporters *e.g.* LAT1 have been identified on the brain capillary endothelial cells which form the BBB. LAT1 is expressed on the luminal and abluminal membranes of the capillary endothelial cells and it efficiently transports neutral L-amino acids (*e.g.*, phenylalanine and leucine) into the brain. Amino acid transporters are also expressed in the neuronal and other cells in the brain

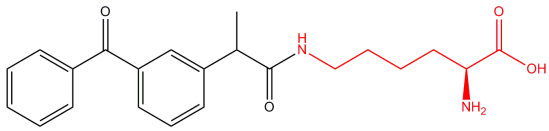
parenchyma (Boado et al., 1999; Duelli et al., 2000). The exploitation of the LAT1 with a prodrug approach could be an innovative way to enhance BBB permeation properties (Sakaeda et al., 2001; Killian et al., 2007; Gynther et al., 2008) and improve drug uptake into the brain intracellular compartment (Su et al., 1995; Wang and Welty, 1996) without increasing non-specific tissue binding. In addition, if one could achieve selective release of the active drug in the target tissue, this would result in a reduced plasma concentration of the drug and fewer peripheral effects.

The aim of the study was to design and synthesize an amino acid prodrug of ketoprofen and to evaluate its ability to cross the BBB via LAT1. The mechanism of brain uptake of the prodrug was determined with *in situ* rat brain perfusion technique. In addition, the ability deliver ketoprofen into the brain ICF *in vivo* was determined. For the *in vivo* brain uptake evaluation i.v. bolus injection method was used. Furthermore, the distribution of the prodrug inside the brain parenchyma was evaluated.

6.2 RESULTS AND DISCUSSION

6.2.1 Design of the prodrug

L-Lysine was selected as the promoiety because the amine functional group in the side chain permits the formation of biodegradable linkage between this amino acid and ketoprofen. L-Lysine is not a LAT1 substrate itself because of the hydrophilic amino group in the side chain. However, it was hypothesized that by conjugating the amino group with ketoprofen via an amide bond, the prodrug formed would function as a LAT1 substrate since the hydrophilic amino group had been removed.



Ketoprofen-L-lysine amide (**6**)

Figure 6.1. Chemical structure of ketoprofen-L-lysine amide (**6**).

6.2.2 Chemical and enzymatic stability of the prodrugs

The L-lysine-ketoprofen prodrug (**6**) was stable in aqueous solutions for two months. Therefore, the prodrug demonstrated sufficient chemical stability in aqueous solutions for further evaluation. If the target for a prodrug design is the site-selective drug delivery to the CNS, the prodrug must not only be delivered to its intended site of action, but its bioconversion should also be selective (Anderson, 1996). **6** did not undergo bioconversion in rat serum or tissue homogenates *in vitro*.

6.2.3 Determination of the brain uptake mechanism for prodrugs

The ability of the prodrugs to bind into LAT1 was studied with the *in situ* rat brain perfusion technique. In the presence of 90 μM L-lysine-ketoprofen prodrug, the [^{14}C]L-leucine PA product was significantly decreased from 0.02059 ± 0.00323 mL/s/g (mean ± s.d., n=5) to 0.00426 ± 0.00019 mL/s/g (mean ± s.d., n=3) (79.3% inhibition). This is a clear evidence of significant binding of the prodrug to the LAT1 (Fig. 6.2). To further study the binding kinetics of the prodrug to LAT1, the PA product of [^{14}C]L-leucine was determined after perfusing rat brain first with the prodrug for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium and finally perfusing the rat brain with 0.2 μCi/mL [^{14}C]L-leucine for 30 s. The PA product of [^{14}C]L-leucine had recovered, indicating that the binding of L-lysine-ketoprofen prodrug to the LAT1 is reversible (Fig. 6.2).

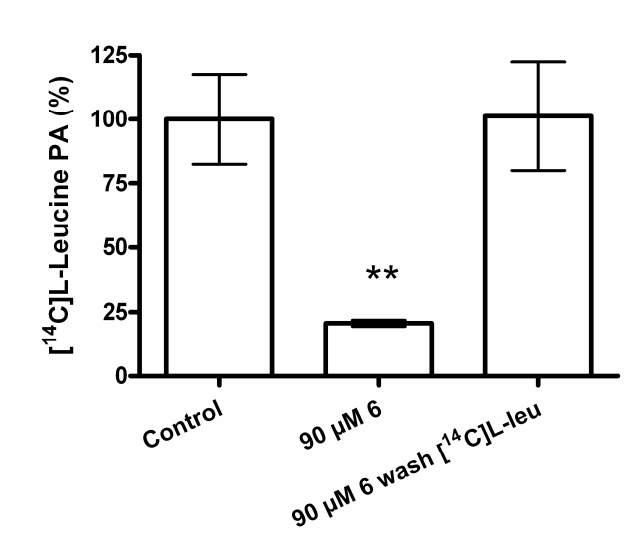


Figure 6.2. Mechanism of rat brain uptake of the prodrugs. The PA product of 0.2 μCi/mL [^{14}C]L-leucine in absence or presence of **6**. The control PA product 0.02059 ± 0.00323 mL/s/g (mean ± s.d., n=5) is decreased to 0 0.00426 ± 0.00019 mL/s/g in the presence of 90 mM **6** (79.3 % inhibition). The uptake of [^{14}C]L-leucine is recovered after washing the prodrug from the brain capillaries demonstrating the PA product of 0.02084 ± 0.0031 mL/s/g for **6**. The data is presented as mean ± s.d. (n=3). An asterisk denotes a statistically significant difference from the respective control (**P<0.01, one-way ANOVA, followed by Dunnett t-test).

After demonstrating affinity for LAT1, the *in situ* rat brain uptake of the prodrug was determined to be concentration dependent in the dose range from 0 to 400 μM for **6** (Fig. 6.3), indicative of transporter-mediated uptake. In addition, the brain uptake of 25 μM **6** was 0.068 ± 0.000 pmol/mg/min which significantly decreased to 0.011 ± 0.020 pmol/mg/min after addition of 2 mM L-phenylalanine, a LAT1 substrate (Kageyama et al., 2000), to the perfusion medium, evidence that the uptake was LAT1-mediated (Fig. 6.4).

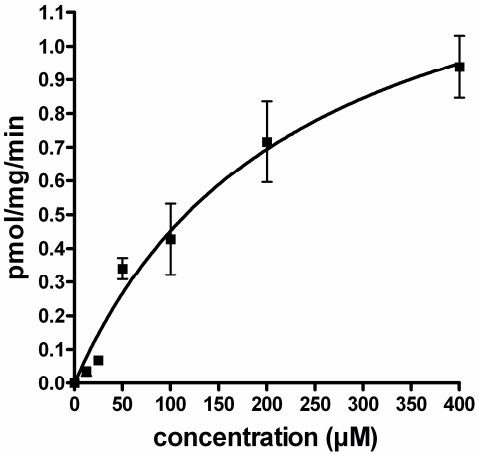


Figure 6.3. Kinetics of the rat brain uptake of the prodrugs. K_m and V_{max} are 231.6 ± 60.4 μM and 1.50 ± 0.20 pmol/mg/min (mean ± s.d., n=3) for **6**.

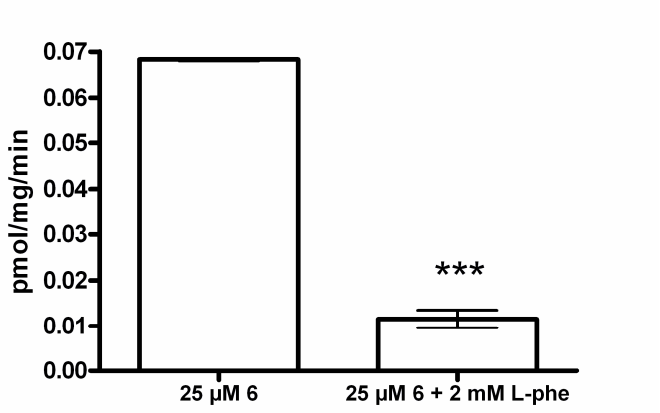


Figure 6.4. The brain uptake of 25 μM **6**. The brain uptake of **6** was 0.068 ± 0.000 pmol/mg/min which significantly decreased to 0.011 ± 0.02 pmol/mg/min after addition of 2 mM L-phenylalanine. The data is presented as mean ± s.d. (n=3). The decrease of the uptake is statistically significant (***P=0.001, t-test).

6.2.4 Ketoprofen-lysine amide is able to cross rat BBB *in vivo* and is rapidly distributed from the brain extracellular compartment

Encouraged by the *in situ* results, we determined the ability of **6** cross the BBB *in vivo* and compared its uptake to that of ketoprofen. 0.2 mL of 30 mM (0.6 μmol) **6** or ketoprofen solution was administered into the left jugular vein in rats. Samples were collected with microdialysis probes from femoral vein and striatum for 300 min: first at 10 min intervals for 60 min and then at 20 min intervals. **6** could be detected from the brain ECF, evidence that **6** is able to cross the BBB *in vivo*. The area under the concentration curve for unbound drug (AUC_u 10-300 min) in brain ECF and plasma was determined for ketoprofen, **6**, and ketoprofen released from **6** (Table 6.2). While the apparent maximum concentrations of **6** in both brain and blood were achieved rapidly, this was followed by a rapid distribution. In contrast, ketoprofen achieved the apparent maximum concentration in the brain slower (Fig. 6.5 a and b). The high free concentration of **6** compared to total plasma concentration suggests that **6** is not highly bound to plasma proteins. This was confirmed by measuring the free fraction ($f_{u,plasma}$) of ketoprofen and **6** in rat plasma *in vitro* (Table 6.1). However, the $f_{u,plasma}$ of ketoprofen after its administration is approximately 60-fold higher compared to the $f_{u,plasma}$ of ketoprofen released from **6**. The *in vitro* $f_{u,plasma}$ data is consistent with the ketoprofen *in vivo* data. Therefore, it is possible that the lower $f_{u,plasma}$ of ketoprofen released from **6** is an artifact caused by analytical difficulties due to low ketoprofen concentrations in rat plasma. However, it is now clear that **6** is able to penetrate the brain, but it was still unclear why **6** is so rapidly eliminated from the ECF. After i.v. injection of **6** only a small concentration of released ketoprofen was detected in plasma and the levels were barely detectable in ECF (Table 6.1), indicating that the rapid elimination of **6** from the ECF is not due to metabolism of **6**.

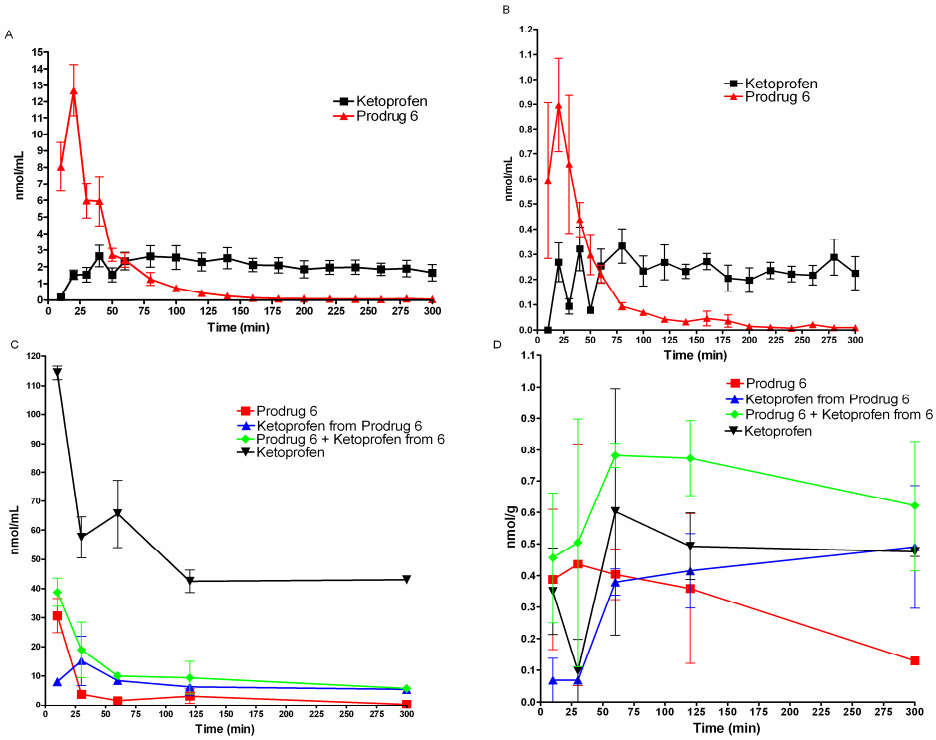


Figure 6.5. *In vivo* pharmacokinetics and brain uptake of **6** and ketoprofen after i.v. bolus injection. (A) Free concentration of **6** and ketoprofen in rat blood after 6 μmol i.v. bolus injection of **6** or ketoprofen (B). Free concentration of **6** and ketoprofen in rat brain ECF after 6 μmol i.v. bolus injection of **6** or ketoprofen. (C) The plasma concentration of **6**, ketoprofen released from **6** and ketoprofen after administration of **6** or ketoprofen. (D) The brain concentration of **6**, ketoprofen released from **6** and ketoprofen after administration of **6** or ketoprofen. The concentrations are presented as mean ± s.e.m (n=3-7).

6.2.5 Ketoprofen-lysine amide is actively transported into the brain cells

The microdialysis results indicated that there was either active efflux of **6** from brain to blood, or active influx from ECF to brain cells. In addition, high non-specific binding into the brain

tissue may also have accounted for the rapid decrease in the ECF concentrations. In an attempt to elucidate which of these mechanisms was involved, the concentrations of **6** and ketoprofen were determined from whole brain tissue and blood after an i.v. bolus injection of **6** or ketoprofen (Fig. 6.5 c and d). After the injection, the rats were sacrificed at specific time points ranging from 10 to 300 min. Blood and brain samples were analyzed for **6** and ketoprofen concentrations (Table 6.1). The results support the proposal that **6** is transported into the brain. In addition, the results indicate that ketoprofen is released from **6** within the brain tissue. When whole tissue results are combined *in vitro* to the result acquired from the microdialysis studies, the distributions of **6** and ketoprofen inside the BBB, the value of $V_{u,brain}$, can be determined (Table 6.1). The higher $V_{u,brain}$ of **6** compared to that of ketoprofen indicates that **6** is rapidly removed from the ECF into the cells, and not effluxed into the blood circulation. However, the high $V_{u,brain}$ may also suggest that **6** is highly bound non-specifically within the brain tissue, which is not desirable. Therefore, the free fractions in brain tissue of **6** and ketoprofen were determined *in vitro* (Table 6.1). **6** and ketoprofen were found to have quite similar free fractions in brain tissue, indicating that the larger $V_{u,brain}$ of **6** is not due to non-specific binding to brain tissue. In addition, the concentration ratio of unbound drug between ECF and ICF was calculated (Table 6.1). Previously, uptake of **6** across the BBB was shown to be LAT1-mediated in the *in situ* experiments. Furthermore, the PSA of **6** is 109.5 $Å^2$, which is higher than the proposed maximum PSA of molecules that can readily penetrate cell membrane via passive diffusion (Pajouhesh and Lenz, 2005). Therefore, there is significant body of evidence supporting the proposal that uptake of **6** into the brain cells is transporter-mediated.

Table 6.1. Brain uptake kinetics and brain distribution of ketoprofen, **6**, and ketoprofen released from **6**.

	Ketoprofen	Ketoprofen **6**	**6**	Ketoprofen from **6** and **6** compared to ketoprofen[a]
$V_{u,\ brain}$	1.9 mL/g	-	5.0 mL/g	-/2.6
$f_{u,\ brain\ in\ vitro}$	0.24	-	0.19	-/0.79
$f_{u,\ plasma\ in\ vitro}$	0.04	-	0.28	-/7
AUC_{brain}[b]	134.1 nmol/g×min	113.7 nmol/g×min	203.3 nmol/g×min	0.8/1.5
$AUC_{brain\ ECF}$[b]	70.8 nmol/mL×min	0.5 nmol/mL×min	40.5 nmol/mL×min	0.007/0.6
AUC_{plasma}[b]	14507 nmol/mL×min	2128 nmol/mL×min	3162 nmol/mL×min	0.15/0.22
$AUC_{u,plasma}$[b]	605.0 nmol/mL×min	1.5 nmol/mL×min	455.7 nmol/mL×min	0.002/0.8
AUC_{cell}[b]	199.9 nmol/mL_{cell}×min	189.3 nmol/mL_{cell}×min	325.3 nmol/mL_{cell}×min	0.95/1.63
$AUC_{u,cell}$[b]	33.5 nmol/mL_{ICF}×min	31.8 nmol/mL_{ICF}×min	48.7 nmol/mL_{ICF}×min	0.95/1.45
$AUC_{u,cell}$/ $AUC_{u,brainECF}$[b]	0.47	63.6	1.20	135/2.6
$AUC_{u,cell}/AUC_{u,plasma}$[b]	0.06	21.8	0.11	363/1.8

[a] *Ratio of values of ketoprofen released from* **6** *and values of* **6** *compared to values of ketoprofen itself.*

[b] *AUC-values are calculated as 10-300 min.*

6.2.6 Ketoprofen-lysine amide is able to release the parent drug at the site of action

The results indicating, that **6** is able to cross the BBB via LAT1 and is subsequently actively transported into brain cells *in vivo*, encouraged us to compare the abilities of **6** and ketoprofen to deliver unbound ketoprofen into the brain ICF. The area under the concentration curve of unbound drug in the ICF ($AUC_{u,cell}$) was determined for ketoprofen, **6** and ketoprofen released from **6** (Table 6.1). The $AUC_{u,cell}$ values of ketoprofen and ketoprofen released from **6** were almost identical. However, at 300 min there was still **6** present in the ICF, which probably is able to release more ketoprofen at the later time-points. In addition, the distribution of ketoprofen originating from **6** and ketoprofen between ICF and ECF was calculated (Table 6.1). Since ketoprofen is released from **6** intracellularly, the ICF-to-ECF ratio was significantly (135 times) higher for 6 as compared to ketoprofen administration. Furthermore, it has been reported that the relationship between brain unbound ECF concentration and unbound plasma concentration is the most useful parameter in the evaluation of the extent of the brain drug delivery (Hammarlund-Udenaes et al., 2008; Liu et al., 2008). However, when there is active transport present from ECF to ICF, it cannot be assumed that the ECF and ICF concentrations are equal. In addition, the target protein of ketoprofen resides within the cell (Spencer et al., 1998; Teismann et al., 2003). Therefore, the more appropriate parameter is the unbound concentration ratio between ICF and plasma. The results show that the concentration ratio of ketoprofen when **6** is administered is 363 times larger than that obtained after ketoprofen administration. This is due to low ketoprofen concentration released from **6** in plasma and the better brain uptake of **6** than can be achieved with ketoprofen. However, as the $AUC_{u,cell}$ of L-lysine-ketoprofen prodrug is not significantly higher compared to that of ketoprofen, the 363-fold difference in unbound ICF-to-plasma ratio has to be due to lower ketoprofen concentrations in plasma. The lower plasma concentration of ketoprofen after **6**

administration is due to higher volume of distribution (V_d) and faster elimination of **6** from central compartment, compared to ketoprofen. The V_d and C_L were calculated for both **6** and ketoprofen after i.v. bolus injection. The V_d and C_L were 0.046 L and 0.017 L/min for **6** and, 0.028 L and 0.001 L/min for ketoprofen, respectively.

6.2.7 Data Analyses

Statistical differences between groups were tested using one-way ANOVA, followed by two-tailed Dunnett t-test (Fig. 6.2). In figure 6.3, two tailed independent samples t-test was used. $P<0.05$ was considered as statistically significant. Data analyses for the dose-uptake curve (Fig. 6.4) were calculated as nonlinear regressions using GraphPad Prism 4.0 for Windows. The area under the concentration curves were calculated as 10-300 min, because there were too few data points in the elimination phase to be extrapolated as 0-∞. The area under the concentration curves were calculated with GraphPad Prism 4.0 for Windows. The normality of the data was tested using Shapiro-Wilk test. All statistical analyses were performed using SPSS 14.0 for Windows.

6.3 CONCLUSIONS

In the present study we were able to demonstrate that amino acid prodrugs of ketoprofen are able to cross the BBB LAT1-mediatedly. In addition, our results supported the earlier proposal that the parent drug has to be conjugated with the amino acid promoiety from the side chain in order to maintain the affinity for LAT1. The synthesized prodrugs **6**, was able to utilize LAT1 for brain permeation. Our results show that **6** was able to significantly inhibit the LAT1-mediated uptake of L-leucine, the brain uptake of the prodrug was saturable and the uptake was inhibited by LAT1 substrate. According to *in situ* brain uptake studies **1** has higher affinity for LAT1 compared to

6 (Fig. 5.2 and 6.2). This may be due to higher lipophilicity of **1** or to the 3D structure of the prodrugs. However, **1** was too labile to be tested *in vivo*. Therefore, the *in vivo* brain uptake was determined only for **6**.

In the *in vivo* studies we were able to demonstrate that **6** is able to cross the BBB. In addition, a large fraction of the prodrug was taken up by brain cells, where ketoprofen was released. In fact, **6** acts rather similarly as another LAT1 substrate, gabapentin, a drug which has low lipophilicity and is extensively distributed into brain cells (Friden et al., 2007). In addition, several authors have reported that gabapentin is actively transported into neuronal cells (Su et al., 1995; Wang and Welty, 1996). The high free fraction of **6** in plasma and the rapid cellular uptake into the brain cells indicate that there is a steep concentration gradient across the BBB, which enables LAT1-mediated uptake of **6** across the BBB. In addition, **6** was rapidly removed from ECF into ICF where a significant amount of ketoprofen was released. Furthermore, the distribution of unbound ketoprofen at the active site compared to plasma concentration was 363 times larger when **6** was administered compared to the corresponding situation with ketoprofen. This is probably due to distribution of **6** into peripheral tissues. However, the concentrations of **6** and ketoprofen should be determined from peripheral tissues to confirm this. In addition, the pharmacokinetics of **6** could be elucidated more using i.v. infusion to achieve steady state. Although the present study evaluated only the brain uptake of this ketoprofen prodrug, the strategy may offer a potential way to achieve shuttling of other small molecular weight CNS drugs especially with low BBB permeation, into the brain. In conclusion, conjugating ketoprofen with L-lysine, LAT1-mediated brain uptake and drug distribution into the ICF of brain parenchyma can be achieved.

6.4 REFERENCES

Anderson B D: Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19: 171-202, 1996.

Boado R J, Li J Y, Nagaya M, Zhang C, Pardridge W M: Selective expression of the large neutral amino acid transporter at the blood-brain barrier. Proc Natl Acad Sci U S A 96: 12079-12084, 1999.

de Lange E C and Danhof M: Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin Pharmacokinet 41: 691-703, 2002.

Duelli R, Enerson B E, Gerhart D Z, Drewes L R: Expression of large amino acid transporter LAT1 in rat brain endothelium. J Cereb Blood Flow Metab 20: 1557-1562, 2000.

Friden M, Gupta A, Antonsson M, Bredberg U, Hammarlund-Udenaes M: In vitro methods for estimating unbound drug concentrations in the brain interstitial and intracellular fluids. Drug Metab Dispos 35: 1711-1719, 2007.

Gynther M, Laine K, Ropponen J, Leppanen J, Mannila A, Nevalainen T, Savolainen J, Jarvinen T, Rautio J: Large neutral amino acid transporter enables brain drug delivery via prodrugs. J Med Chem 51: 932-936, 2008.

Hammarlund-Udenaes M, Friden M, Syvanen S, Gupta A: On the rate and extent of drug delivery to the brain. Pharm Res 25: 1737-1750, 2008.

Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N, Shimohama S: The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. Brain Res 879: 115-121, 2000.

Killian D M, Hermeling S, Chikhale P J: Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. Drug Deliv 14: 25-31, 2007.

Liu X, Chen C, Smith B J: Progress in brain penetration evaluation in drug discovery and development. J Pharmacol Exp Ther 325: 349-356, 2008.

Pajouhesh H and Lenz G R: Medicinal chemical properties of successful central nervous system drugs. NeuroRx 2: 541-553, 2005.

Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, Savolainen J: Prodrugs: design and clinical applications. Nat Rev Drug Discov 7: 255-270, 2008.

Sakaeda T, Tada Y, Sugawara T, Ryu T, Hirose F, Yoshikawa T, Hirano K, Kupczyk-Subotkowska L, Siahaan T J, Audus K L, Stella V J: Conjugation with L-Glutamate for in vivo brain drug delivery. J Drug Target 9: 23-37, 2001.

Spencer A G, Woods J W, Arakawa T, Singer, II, Smith W L: Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. J Biol Chem 273: 9886-9893, 1998.

Su T Z, Lunney E, Campbell G, Oxender D L: Transport of gabapentin, a gamma-amino acid drug, by system l alpha-amino acid transporters: a comparative study in astrocytes, synaptosomes, and CHO cells. J Neurochem 64: 2125-2131, 1995.

Summerfield S G, Read K, Begley D J, Obradovic T, Hidalgo I J, Coggon S, Lewis A V, Porter R A, Jeffrey P: Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. J Pharmacol Exp Ther 322: 205-213, 2007.

Summerfield S G, Stevens A J, Cutler L, del Carmen Osuna M, Hammond B, Tang S P, Hersey A, Spalding D J, Jeffrey P: Improving the in vitro prediction of in vivo central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. J Pharmacol Exp Ther 316: 1282-1290, 2006.

Teismann P, Tieu K, Choi D K, Wu D C, Naini A, Hunot S, Vila M, Jackson-Lewis V, Przedborski S: Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. Proc Natl Acad Sci U S A 100: 5473-5478, 2003.

Wang Y and Welty D F: The simultaneous estimation of the influx and efflux blood-brain barrier permeabilities of gabapentin using a microdialysis-pharmacokinetic approach. Pharm Res 13: 398-403, 1996.

7 Glucose promoiety enables GluT-1 -mediated brain uptake of ketoprofen and indomethacin prodrugs

Abstract. GluT1 is present both on the luminal and the abluminal membrane of the endothelial cells forming the BBB. One attractive approach to utilize GluT1 for enhanced brain drug delivery of CNS drugs is to conjugate an endogenous transporter substrate to the active drug molecule to utilize the prodrug approach. In the present study, ketoprofen and indomethacin were conjugated with glucose and the brain uptake mechanism of the prodrugs was determined with the *in situ* rat brain perfusion technique. Two of the four prodrugs were able to significantly inhibit the GluT1-mediated uptake of glucose, thereby demonstrating affinity to the transporter. Furthermore, the prodrugs were able to cross the BBB in a temperature dependent manner, suggesting that the brain uptake of the prodrugs is carrier-mediated. These results indicate that glucose prodrugs are able to cross the BBB via GluT1.

Adapted with permission of the American Chemical Society from: Gynther M., Ropponen J., Laine K., Leppänen J., Haapakoski P., Peura L., Järvinen T., Rautio J.: Glucose Promoiety Enables Glucose Transporter Mediated Brain Uptake of Ketoprofen and Indomethacin Prodrugs in Rats. Journal of Medicinal Chemistry 52: 3348-3353, 2009.

7.1 INTRODUCTION

GluT1 is present both on the luminal and the abluminal membrane of the endothelial cells forming the BBB (Farrell, Pardridge, 1991). GluT1 transports glucose and other hexoses and has the highest transport capacity of the carrier-mediated transporters present at the BBB, being therefore an attractive transporter for prodrug delivery (Anderson, 1996). Several *in vitro* studies have been performed with different drug molecules in order to determine the ability of glycosyl derivatives to bind to GluT1. In addition, systemically delivered glycosyl derivatives of 7-chlorokynurenic acid, L-dopa, and dopamine have been shown to have pharmacological activity in the CNS of rodents (Halmos et al., 1996; Battaglia et al., 2000; Bonina et al., 2003; Fernandez et al., 2003). These previous studies have indeed demonstrated that glucose conjugates can bind to GluT1 and that the derivatives are centrally available, but none of these studies verified the ability of conjugates to cross the BBB via GluT1.

The overall aim of the present study was to show with two model compounds ketoprofen and indomethacin using *in situ* rat brain perfusion technique, that GluT1 can be utilized to carry non-substrate drugs into the brain by conjugating a drug molecule to D-glucose with bioreversible linkage. Four glucose prodrugs synthesized and studied are ketoprofen-glucose produg (**7**), indomethacin-glucose prodrug (**8**), indomethacin-glycolic acid-glucose prodrug (**9**) and indomethacin-lactic acid-glucose prodrug (**10**) (Fig. 7.1).

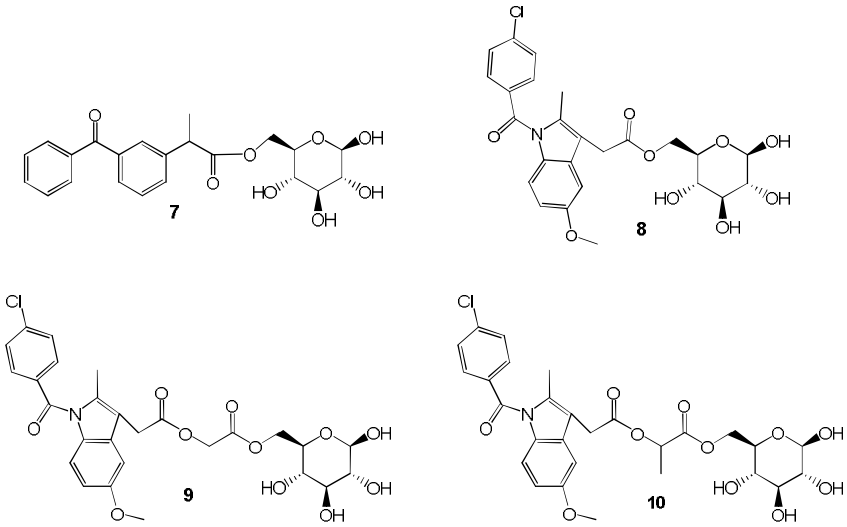


Figure 7.1. Chemical structures of ketoprofen prodrug **7** and indomethacin prodrugs **8**, **9** and **10**.

7.2 RESULTS AND DISCUSSION

7.2.1 Design of the prodrugs

Ketoprofen and indomethacin were chosen as model compounds because they are easy to detect with UV-detector and they have a carboxyl group, which makes it easy to conjugate with D-glucose. In addition, it has been proposed that non-steroidal anti-inflammatory drugs may have some therapeutic effects in CNS disorders, such as Alzheimer`s disease. The molecular weights of ketoprofen and indomethacin are 254 Da and 357 Da, respectively. Therefore, the ability of GluT1 to transport different sized molecules can be evaluated.

According to the GluT1 model published by (Mueckler, Makepeace, 2008) the hydroxyl group of glucose situated at the carbon 6-position goes into a hydrophobic pocket in the transporter protein substrate binding site. In addition, the hydroxyl group at the carbon 6-position does not form a hydrogen bond with the transporter that would be crucial for the affinity. Fernandez et al., (2000) synthesized several glycosyl

derivatives of dopamine and tested the affinity of the prodrugs to GluT1 in human erythrocytes. Dopamine was linked to glucose with different linkers at the carbon 1-, 3-, and 6-positions of glucose. The results of glucose uptake inhibition showed that the glucose derivatives that were conjugated at position 6 had the best affinity for GluT1. Therefore, the hydroxyl group at the carbon 6-position is likely the most potential functional group to which to attach the drug molecule in order to maintain the affinity of the glucose conjugate for GluT1.

7.2.2 Chemical and enzymatic stability of prodrugs

The degradation of prodrugs **7** - **10** was studied in aqueous buffer solution of pH 7.4 at 37 °C. The degradation of **7** and **8** followed pseudo-first-order kinetics with the half-lives of 88.6 ± 0.0 and 73.4 ± 1.6 hours (mean ± s.d., n=2), respectively (table 6.1). Therefore, both **7** and **8** demonstrated sufficient chemical stability in aqueous solutions for further evaluation. However, **9** and **10** were degraded with the half-lives of 3.0 ± 0.2 and 9.5 ± 0.0 hours (mean ± s.d., n=2). Therefore, **9** and **10** are not stable enough to be tested with *in situ* rat brain perfusion technique.

7 was susceptible to enzymatic hydrolysis as it was quantitatively cleaved to ketoprofen and glucose in 20% rat brain homogenate and in 50% rat liver homogenate. The enzymatic hydrolysis followed pseudo-first-order kinetics, the half-lives being 43.5 ± 3.6 and 8.6 ± 0.6 minutes (mean ± s.d., n=3) in brain homogenate and liver homogenate, respectively. Therefore, **7** undergoes bioconversion to ketoprofen and glucose in the brain tissue. However, **7** is also highly susceptible to enzymatic hydrolysis in liver which may compromise its effective brain drug delivery *in vivo*. Enzymatic hydrolysis studies of **8** in neither brain nor liver homogenate resulted in the formation of indomethacin which makes the prodrug not suitable for brain delivery. Since indomethacin was not released from the prodrug, the hydrolysis products and the half-life of **8** were not identified. In an attempt to solve the problem, we

synthesised two prodrugs (**9** and **10**) with glycolic acid and lactic acid linker group between glucose and indomethacin. However, these were not stable enough to be tested.
Although **8** did not release the parent drug in brain tissue, it is reasonable to determine the brain uptake of **8**. Since **8** has higher molecular weight than **7**, determining the uptake mechanism of both prodrugs may give more insight into the GluT1 ability to transport molecules across the BBB.

Table 7.1. Molecular weight, hydrolysis rates in phosphate buffer solution and polar surface area of the prodrugs.

Prodrug	Molecular weight (g/mol)	$t_{1/2}$ (h) 37 °C phosphate buffer (pH 7.4)	Polar surface area (Å^2)
7	416	88.5 ± 0.0	133.52
8	519	40.5 ± 2.5	145.99
9	577	3.0 ± 0.2	172.29
10	591	9.5 ± 0.0	172.29

mean ± s.d., n=2

7.2.3 Determination of the brain uptake mechanism for prodrugs

The *in situ* rat brain perfusion technique has not been previously used to determine the brain uptake of glycosyl conjugates. Therefore, the suitability of the technique for glycosyl conjugate uptake determination was studied by confirming the presence of functional GluT1-transporters in rat BBB with [^{14}C]D-glucose, which is an endogenous substrate for the GluT1 (Farrell, Pardridge, 1991). The uptake of molecules across the BBB was quantified by determining the permeability-surface area (PA) product. The PA product of 0.2 μCi/mL [^{14}C]D-glucose was determined to be 0.0042 ± 0.0002 mL/s/g (mean ± s.d., n=4) (Fig. 7.2). In addition, the determination of cerebrovascular GluT1 functional expression was carried out with a competition assay by perfusing [^{14}C]D-glucose (0.2 μCi/mL) with 20 mM

concentration of glucose. This co-perfusion resulted in a brain uptake of 0.0011 ± 0.0003 mL/s/g (mean ± s.d., n=3) (73.8% inhibition) of [^{14}C]D-glucose, thereby demonstrating functional expression of cerebrovascular GluT1. The PA product was also determined using 5 °C perfusion medium which resulted in 76.2% inhibition of the PA product of [^{14}C]D-glucose to 0.001 ± 0.0003 mL/s/g (mean ± s.d., n=3). This further suggests that the uptake of [^{14}C]D-glucose in the *in situ* rat brain perfusion test method was carrier-mediated, since this type of uptake is reduced when the temperature is lowered (Kageyama et al., 2000; Gynther et al., 2008). However, the uptake of [^{14}C]D-glucose is not completely inhibited at low temperature, which suggests that some activity of GluT1 is still present at the BBB. There is also the possibility that a part of the uptake of [^{14}C]D-glucose is due to passive diffusion. However, 80 μM **8** is able to inhibit the [^{14}C]D-glucose uptake almost completely (96.7% inhibition), which indicates that there is no passive uptake of [^{14}C]D-glucose present at the BBB.

The ability of **7** and **8** to bind into GluT1 was studied by co-perfusing increasing concentrations of the prodrugs with [^{14}C]D-glucose. The prodrugs were able to inhibit the uptake of [^{14}C]D-glucose in a concentration-dependent manner (Fig. 7.3). Non-linear regression analysis was used to determine the half-maximal inhibitory concentration (IC50) values of **7** and **8**. The IC50 values were 32.85 ± 8.17 μM for **7** and 0.71 ± 0.04 μM for **8**, which indicates that **7** has lower affinity for GluT1 compared to **8**.

To further study the binding kinetics of the prodrugs to GluT1, the PA product of [^{14}C]D-glucose was determined after perfusing rat brain first with the prodrugs at 80 μM for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium and finally perfusing the rat brain with 0.2 μCi/mL [^{14}C]D-glucose for 30 s. This resulted in the PA products of [^{14}C]D-glucose 0.0033 ± 0.0001 mL/s/g and 0.0032 ± 0.0003 mL/s/g (mean ± s.d., n=3) for **7** and **8**, respectively, indicating that the binding of **7** and **8** to the GluT1 is reversible (Fig. 7.2).

These results show that **8** has higher affinity for GluT1 than **7**, and both of the prodrugs have higher affinity for GluT1 than D-glucose. This higher inhibition caused by the prodrugs compared to D-glucose could be due to the higher molecular weight of the prodrugs. It is proposed that as the substrate binds to the GluT1 binding site, the transporter protein cavity occludes the bound substrate and then opens at the opposite side of the membrane where the bound substrate can dissociate (Blodgett, Carruthers, 2005). The sterical hindrance caused by the higher molecular weight of the prodrugs could slow the conformational change of the transporter, and as the transporters are occupied by the prodrugs, the transporters are not able to facilitate the uptake of [^{14}C]D-glucose. Therefore, the low IC50 values only suggest that the prodrugs are able to bind to GluT1 and the ability cross the BBB is not necessarily achieved.

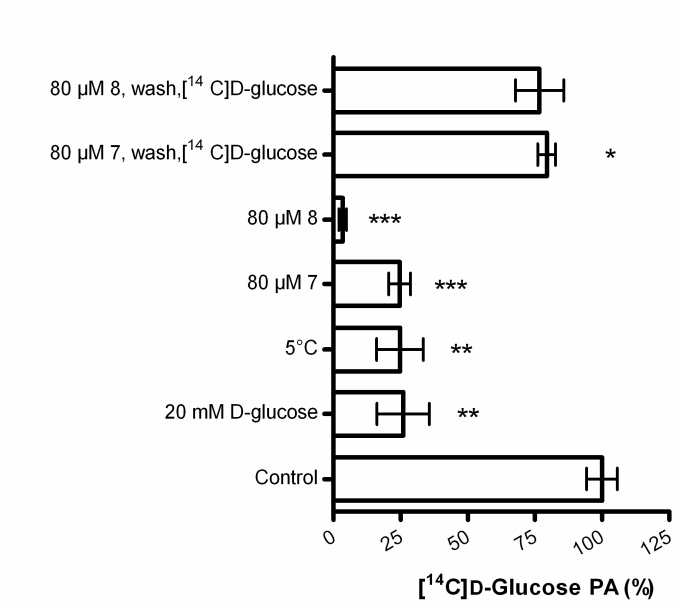


Figure 7.2. Mechanism of **7** and **8** rat brain uptake. The PA product of 0.2 µCi/mL [^{14}C]D-glucose in absence or presence of D-glucose, low temperature, **7** or **8**. The control PA 0.0042 ± 0.0002 mL/s/g (mean ± s.d., n=4) is decreased to 0.0011 ± 0.0003

(mean ± s.d., n=3) (73.8% inhibition) in the presence of 20 mM D-glucose and to 0.001 ± 0.0003 mL/s/g (mean ± s.d., n=3) (76.2% inhibition), when using 5 °C perfusion medium. The PA product of [^{14}C]D-glucose decreased to 0.00103 ± 0.00014 mL/s/g (75.5% inhibition) and 0.00014 ± 0.00005 mL/s/g (96.7% inhibition) (mean ± s.d., n=3) after perfusing the brain with **7** and **8**, respectively. After washing **7** from the brain capillaries the PA product of 0.2 μCi/mL [^{14}C]D-glucose was 0.0033 ± 0.0001 mL/s/g (mean ± s.d., n=3) and the PA product of 0.2 μCi/mL [^{14}C]D-glucose was 0.0032 ± 0.0003 mL/s/g (mean ± s.d., n=3) after **8** was washed from the brain capillaries. An asterisk denotes a statistically significant difference from the respective control (***P<0.001, **P<0.01, *P<0.05, Brown Fortsythe, followed by Dunnett T3-test).

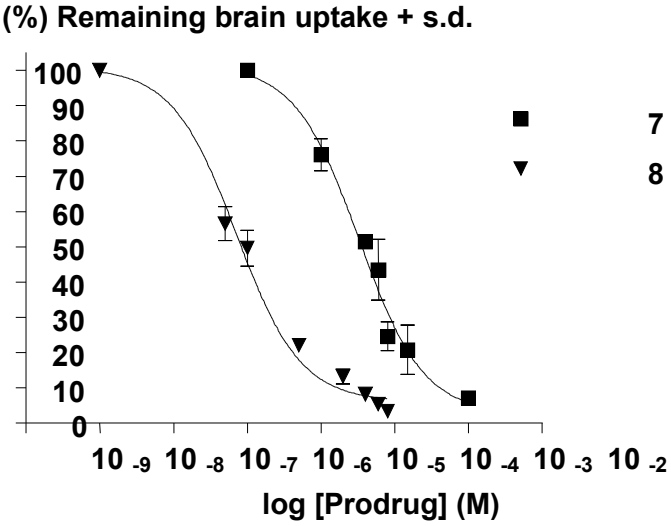


Figure 7.3. Inhibition of 0.2 μCi/mL [^{14}C]D-glucose uptake by **7** and **8**. IC50 values are 32.85 ± 8.17 μM and 0.71 ± 0.04 μM for **7** and **8**, respectively. Data are mean ± s.d. (n = 2). IC50 values are calculated with non-linear regression analysis, using GraphPad Prism 4.0 for Windows.

7.2.4 Brain uptake determination of the prodrugs

In addition to determining the binding of the prodrugs to GluT1, we also determined the brain uptake of the prodrugs across the BBB. The brain uptake of the prodrugs was determined with the *in situ* rat brain perfusion technique using 150 μM and 60 s perfusion time (Fig. 7.4). The perfusion of 150 μM both **7** and **8** indeed resulted in detectable amounts of both prodrugs in brain tissue. The brain uptake of 150 μM 7 was 1.33 ± 0.18 pmol/mg/min (mean ± s.d., n=3), and an addition of 50 mM D-glucose to the perfusion medium decreased the brain uptake 61.4% to 0.513 ± 0.009 pmol/mg/min (mean ± s.d., n=3) (Fig. 7.4). Finally, brain uptake of **7** was determined at 150 μM concentration in a 5 °C perfusion medium. Lower temperature decreased the uptake of **7** 61.3% to 0.515 ± 0.065 pmol/mg/min (mean ± s.d., n=3). The brain uptake of **7** was also determined with 450 μM, 1000 μM, 3500 μM and 15000 μM concentrations but the uptake was not saturable within this concentration range (Fig. 7.5). It is possible that because of poor affinity of **7** for GluT1 and high capacity of the transporter (K_m value of D-glucose uptake is 11 mM and V_{max} is 1420 nmol/min/g), (Anderson, 1996) the uptake did not become saturated, although the uptake of **7** was mainly carrier-mediated. Another explanation for the lack of uptake saturation could be that passive diffusion of **7** is significant enough to hide the saturation of GluT1-mediated uptake.

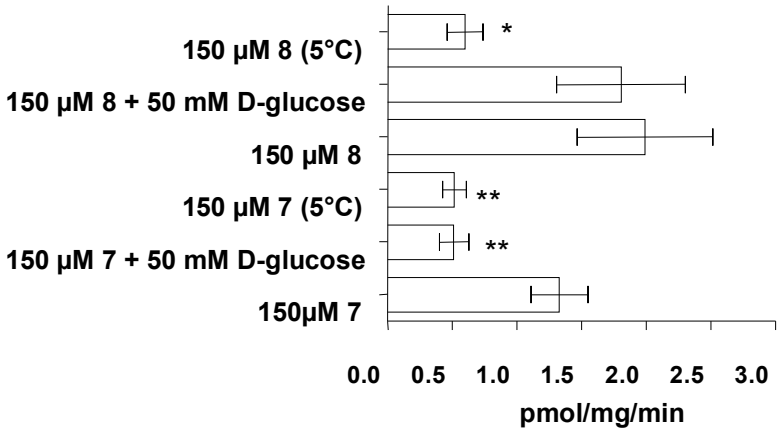


Figure 7.4. The brain uptake of **7** and **8**. The uptake of **7** with 150 µM concentration is 1.33 ± 0.18 pmol/mg/min and addition 50 mM D-glucose to the perfusion medium decreased significantly (61.4% inhibition) the brain uptake to 0.513 ± 0.009 pmol/mg/min (mean ± s.d., n=3). 5 °C perfusion medium decreased significantly the uptake of **7** to 0.515 ± 0.065 pmol/mg/min (mean ± s.d., n=3) (61.3% inhibition). The brain uptake of 150 µM **8** was 1.993 ± 0.429 pmol/mg/min (mean ± s.d., n=3) and after the addition of 50 mM D-glucose to the perfusion medium the uptake was 1.806 ± 0.353 pmol/mg/min (mean ± s.d., n=3). The decrease of the perfusion medium temperature to 5 °C resulted in brain uptake of 0.599 ± 0.098 pmol/mg/min (mean ± s.d., n=3) (69.9% inhibition). An asterisk denotes a statistically significant difference from the respective control (**P<0.01, *P<0.05, one-way ANOVA, followed by Dunnett t-test). Columns 150 µM **7** and 150 µM **8** were used as controls in Dunnett t-test.

The brain uptake of 150 µM **8** was 1.993 ± 0.429 pmol/mg/min (mean ± s.d., n=3) (Fig. 7.4) and after the addition of 50 mM D-glucose to the perfusion medium the uptake was 1.806 ± 0.353 pmol/mg/min (mean ± s.d., n=3). The decrease of the perfusion medium temperature to 5 °C resulted in brain uptake of 0.599 ±

0.098 pmol/mg/min (mean ± s.d., n=3) (69.9% inhibition). The uptake of **8** was slightly higher than the uptake of **7**, and the uptake was not decreased by addition of D-glucose in the perfusion medium, unlike the uptake of **7**. Since addition of D-glucose did not affect the uptake of **8**, as uptake is either due to passive diffusion or the affinity of **8** for GluT1 is much higher than the affinity of D-glucose.

The passive diffusion of the prodrugs across the BBB is probably limited, since the polar surface areas of the prodrugs are over 90 $Å^2$ (Table 6.1) (Pajouhesh, Lenz, 2005). In addition, the uptake of both prodrugs was significantly decreased by low temperature, which indicates that the uptake of the prodrugs is carrier-mediated. The low temperature could not inhibit the uptake of the prodrugs entirely, which may indicate that the brain uptake of the prodrugs is partly due to passive diffusion. However, the uptake of [^{14}C]D-glucose was not entirely inhibited by low temperature and leaving some transporter activity present at the BBB. Therefore, the fraction of passive diffusion of the whole brain uptake cannot be determined without using a non-competing GluT1 inhibitor. As far we are aware, such an inhibitor suitable for *in situ* rat brain perfusion does not exist.

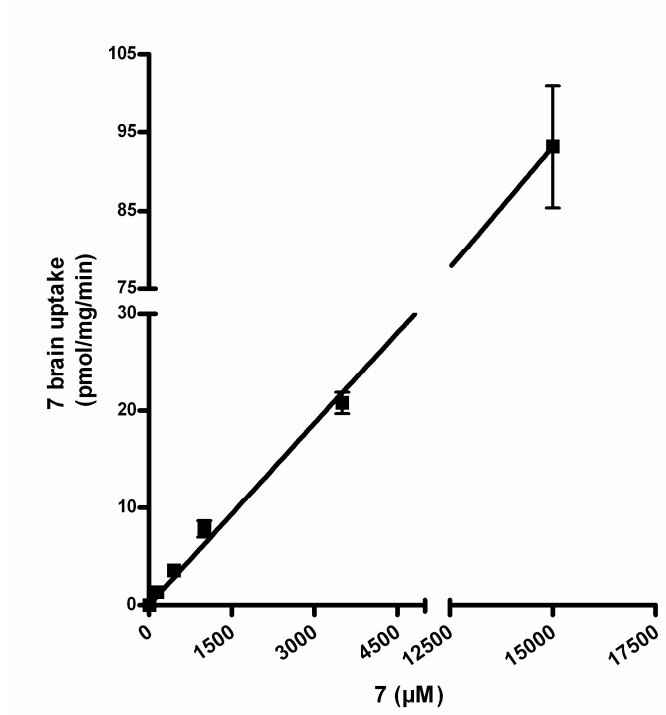


Figure 7.5. Relationship between concentration of the perfusion medium and brain uptake of prodrug **7**. The uptake of **7** was 1.33 ± 0.18, 3.55 ± 0.33, 7.78 ± 0.68, 20.77 ± 0.78 and 93.21 ± 5.51 pmol/mg/min (mean ± s.d., n=3) using 150 μM, 450 μM, 1000 μM, 3500 μM and 15000 μM concentrations, respectively.

7.2.5 Capillary depletion analysis

Capillary depletion analysis of brain samples from perfused brain showed that the prodrugs are present in the supernatant fraction which consists of brain parenchyma (Table 7.2). The concentration of the prodrugs in the endothelial cell enriched pellet fraction was below the lower limit of detection. However, since the uptake of the prodrugs determined from the whole brain is higher compared to brain parenchyma (supernatant), a fraction of the prodrugs is captured into the endothelial cells.

Table 7.2. Capillary depletion analysis after 60 s perfusion of **7** and **8** with 450 μM concentration, followed by washing the prodrug from the capillaries with 30 s perfusion.

Right cerebrum	V_d (mL/g)
7	
Whole brain	0.0054 ± 0.0002
Supernatant	0.0050 ± 0.0006
Pellet	[a]
8	
Whole brain	0.0155 ± 0.0044
Supernatant	0.0135 ± 0.0052
Pellet	[a]

mean ± s.d., n=3, [a] below lower limit of detection

7.2.6 Data Analyses

The results from the brain uptake experiments are presented as mean ± s.d. of at least three independent experiments. Statistical differences between groups were tested using Brown Fortsythe, followed by Dunnett T3 –test (Fig. 7.2) and one-way ANOVA, followed by two-tailed Dunnett t-test (Fig. 7.4). The normality of the data was tested using Shapiro-Wilk test. The IC50 values (Fig. 7.3) were determined by non-linear regression analysis using GraphPad Prism 4.0 for Windows. All statistical analyses were performed using SPSS 14.0 for Windows.

7.3 CONCLUSION

In this study, four glucose prodrugs were synthesized and their ability to cross the BBB via GluT1 was determined with *in situ* rat brain perfusion technique for two of the prodrugs (**7** and **8**).

Both **7** and **8** demonstrated reversible concentration dependent inhibition of brain uptake of the radiotracer [^{14}C]D-glucose in the *in situ* rat brain perfusion model, indicating that the prodrugs bind to the GluT1. In addition, three factors strongly favour the claim that the brain uptake of **7** and **8** is GluT1-mediated. First, both prodrugs were able to cross the BBB and gain entry into the brain tissue. Secondly, the uptake of **7** and **8** was decreased significantly when 5 °C perfusion medium was used, indicating, that the uptake is carrier-mediated. Furthermore, the high polar surface areas of **7** and **8** indicate that passive diffusion across the BBB is limited.

The uptake of **7** was decreased when 50 mM concentration of D-glucose was added in to the perfusion medium. In addition, the uptake of **7** did not become saturated even at 15 mM concentration, which suggests that the affinity of **7** for Glut1 is low and the capacity of the transporter is high. It is also possible that passive diffusion of **7** is significant enough to obscure the saturation of Glut1-mediated uptake. In the case of **8**, the uptake did not decrease when 50 mM D-glucose was added into the perfusion medium. However, the unspecific inhibition of GluT1 using a lowered temperature, suggests that the uptake of **8** is, at least partly, GluT1-mediated. The lack of inhibition of **8** brain uptake by 50 mM D-glucose indicates that the affinity of **8** for the transporter is much higher than the affinity of D-glucose or **7**. This hypothesis is further supported by the low IC50-value of **8**.

These results indicate, that a hydrophilic drug can be attached to the hydroxyl group of D-glucose at the carbon 6-position and still maintain the affinity of the D-glucose promoiety for the GluT1 transporter. However, glucose as a promoiety has several limitations. The structure of glucose limits the amount of drug molecules that can be linked with it by biodegradable bonds. In essence, only drugs that bear a carboxyl acid group can be linked without a linker/spacer with glucose with a prodrug bond. In addition, the stability of ester prodrugs in systemic circulation might not be adequate for clinical use. Therefore this prodrug technology demands further development before being

fully applicable to oral drug delivery. In summary, drug molecules as large as indomethacin, can be conjugated with D-glucose and GluT1 is able to mediate the uptake of the conjugate across the BBB into the brain parenchyma.

7.4 REFERENCES

Anderson B D: Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19: 171-202, 1996.

Battaglia G, La Russa M, Bruno V, Arenare L, Ippolito R, Copani A, Bonina F,Nicoletti F: Systemically administered D-glucose conjugates of 7-chlorokynurenic acid are centrally available and exert anticonvulsant activity in rodents. Brain Res 860: 149-156, 2000.

Blodgett D M,Carruthers A: Quench-flow analysis reveals multiple phases of GluT1-mediated sugar transport. Biochemistry 44: 2650-2660, 2005.

Bonina F, Puglia C, Rimoli M G, Melisi D, Boatto G, Nieddu M, Calignano A, La Rana G,De Caprariis P: Glycosyl derivatives of dopamine and L-dopa as anti-Parkinson prodrugs: synthesis, pharmacological activity and in vitro stability studies. J Drug Target 11: 25-36, 2003.

Farrell C L,Pardridge W M: Blood-brain barrier glucose transporter is asymmetrically distributed on brain capillary endothelial lumenal and ablumenal membranes: an electron microscopic immunogold study. Proc Natl Acad Sci U S A 88: 5779-5783, 1991.

Fernandez C, Nieto O, Fontenla J A, Rivas E, de Ceballos M L,Fernandez-Mayoralas A: Synthesis of glycosyl derivatives as dopamine prodrugs: interaction with glucose carrier GLUT-1. Org Biomol Chem 1: 767-771, 2003.

Fernandez C, Nieto O, Rivas E, Montenegro G, Fontenla J A,Fernandez-Mayoralas A: Synthesis and biological studies of glycosyl dopamine derivatives as potential antiparkinsonian agents. Carbohydr Res 327: 353-365, 2000.

Gynther M, Laine K, Ropponen J, Leppanen J, Mannila A, Nevalainen T, Savolainen J, Jarvinen T,Rautio J: Large neutral amino acid transporter enables brain drug delivery via prodrugs. J Med Chem 51: 932-936, 2008.

Halmos T, Santarromana M, Antonakis K,Scherman D: Synthesis of glucose-chlorambucil derivatives and their recognition by the human GLUT1 glucose transporter. Eur J Pharmacol 318: 477-484, 1996.

Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N,Shimohama S: The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. Brain Res 879: 115-121, 2000.

Mueckler M,Makepeace C: Transmembrane segment 6 of the Glut1 glucose transporter is an outer helix and contains

amino acid side chains essential for transport activity. J Biol Chem 283: 11550-11555, 2008.

Pajouhesh H,Lenz G R: Medicinal chemical properties of successful central nervous system drugs. NeuroRx 2: 541-553, 2005.

8 General discussion

The need for new CNS drug targeting strategies is clear, because a significant number of potential CNS drugs fail to achieve efficient concentrations in their site of action. In this research, an attempt was made to enhance the brain uptake of model drugs by utilizing LAT1 and GluT1 transporters present at the BBB with prodrug approach. This strategy is known in the literature. However, there are many unresolved issues concerning the usefulness of this strategy. First, the methods used to determine the mechanism and extent of brain uptake have to be considered carefully. Second, the distribution of the drug or prodrug in the brain tissue is equally important as the systemic pharmacokinetics. In this research an *in situ* rat brain perfusion technique was used to evaluate the mechanism of BBB permeation. *In situ* technique was chosen because it has advantages over *in vivo* and *in vitro* methods. The most important advantage is that the experimental conditions can be easily manipulated. Therefore, saturable and temperature sensitive processes, such as carrier-mediated brain uptake can be evaluated. In addition, with the *in situ* technique, the ability of the molecules to cross the functional BBB can be determined. With an in vitro method, often only the ability to bind to transporters can be determined. In *in vitro* permeation assays the expression of transporters and tight junctions is not equivalent to the *in vivo* situation. Although *in situ* technique is superior when the mechanism of brain uptake is estimated, it cannot be used to evaluate the extent of brain uptake. In addition, the systemic pharmacokinetics cannot be determined. Therefore, the i.v. single bolus injection method was used to determine the ability of one of the prodrugs to cross the BBB *in vivo*. The distribution beyond the BBB is as important as the ability cross the BBB. Therefore, the distribution of unbound prodrug and released parent drug was assessed with a combination of whole

tissue concentrations, ECF concentrations and *in vitro* unbound fraction in brain homogenate.

8.1 SUMMARY OF THE EVALUATION OF AMINO ACID PRODRUGS

Two amino acid prodrugs were designed to meet requirements for LAT1 substrates based on the LAT1 binding site model which indicated that a potential LAT1 substrate should have a positively charged amino group, a negatively charged carboxyl group and a hydrophobic side chain. Both prodrugs were able to bind to LAT1 and cross the BBB via LAT1. We also designed prodrugs, in which the parent drug was conjugated with the amino acid by either the amino group or the carboxyl group necessary for LAT1 recognition. The inability of these prodrugs to inhibit LAT1 confirmed the requirements for LAT1 substrates.

An L-tyrosine prodrug of ketoprofen demonstrated significant reversible inhibition of brain uptake of the radiotracer [^{14}C]L-leucine in the *in situ* rat brain perfusion model, indicating that the prodrug binds to the LAT1. More importantly, the prodrug is able to cross the endothelial cells and penetrate into the brain parenchyma and the brain uptake of the prodrug was both concentration-dependent and saturable. The uptake of the prodrug was also significantly decreased when 5 °C perfusion medium was used indicating the brain uptake of the prodrug is carrier-mediated. In addition, the LAT1 inhibitor BCH significantly decreased the brain uptake of the prodrug. Overall, these results strongly suggest that the rat brain uptake of the prodrug is LAT1-mediated. Importantly, the prodrug is not only recognized but also transported across the rat BBB by LAT1. In the prodrug structure, L-tyrosine acts as a carrier that possesses the essential structural features needed for LAT1-binding. The systemic pharmacokinetics of the prodrug is unknown but the ester bond between ketoprofen and L-tyrosine is probably cleaved mostly by esterases present in the peripheral tissues.

Therefore this prodrug technology demands further development before being fully applicable to oral drug delivery. To overcome the problem of rapid degradation of the bond between the parent drug and the promoiety, a more stable prodrug has to be designed. Instead of designing an ester prodrug it was decided to try amide bond between the parent drug and amino acid. However, LAT1 substrates have an optimal structure for LAT1 binding. Therefore, the side chains LAT1 substrates do not have suitable functional groups for the formation of an amide bond with ketoprofen. However, there are amino acids which are not LAT1 substrates themselves but have suitable functional groups in their side chain. It was hypothesized that by conjugating the hydrophilic functional group of the side chain with the parent drug, the formed prodrug would be a LAT1 substrate. This hypothesis was tested by designing an L-lysine-ketoprofen amide prodrug. The L-lysine-ketoprofen prodrug was able to cross the BBB by utilizing LAT1. In addition, the prodrug was rapidly removed, probably by active transporter, from brain ECF into ICF where ketoprofen was released. Furthermore, the distribution of unbound ketoprofen at the active site compared to plasma concentration was 363 times larger when the prodrug was administered compared to the corresponding situation with ketoprofen. However, the prodrug was probably distributed into peripheral tissues, because the plasma concentrations of the prodrug were low. The most important results are that other amino acids than LAT1 substrates can be used as promoieties and amino acid prodrugs are able to deliver the parent drug into the brain ICF *in vivo*. In addition, the distribution of the prodrugs inside the brain parenchyma can be determined by combining *in vivo* and *in vitro* methods. To further evaluate the potential of this prodrug approach to enhance the brain uptake of drugs, other model drugs should be tested. Preferably the model drugs should have an extremely low *in vivo* brain uptake. In addition to evaluating the usefulness of this prodrug approach with drugs with poor brain uptake, other issues need to be considered. As the brain uptake of the parent drug is changed

after the formation of the prodrug, also the distribution in to other tissues should be evaluated. Although human and rat both express LAT1 at the BBB, there are differences in the amino acid sequence of the transporters. Both species share the same LAT1 substrates which is not unreasonable considering the availability of these amino acids in the diet. However, the ability of both human and rat LAT1 to recognize the same natural amino acids does not mean that both LAT1 variations are able to recognize synthesized amino acids such as amino acid prodrugs. Therefore, the LAT1 mediated brain uptake of prodrugs in rats does not necessary mean that these prodrugs are able to utilize human LAT1. Furthermore, the brain uptake studies in this thesis, as in majority of other studies, have been made with healthy animals. However, the BBB is not fully functional in many CNS disorders. The tight junctions between the endothelial cells may be compromised and molecules are able to penetrate the brain more readily. In addition, the expression and function of influx or efflux transporters may be changed, which may increase or decrease the brain uptake of the transporter substrates. Therefore, if possible the brain uptake studies should also be done with animals, which are affected by the CNS disorder that the prodrug is designed to treat.

8.2 SUMMARY OF THE EVALUATION OF GLUCOSE PRODRUGS

Four glucose prodrugs were synthesized and their ability to cross the BBB via GluT1 was determined for two of the prodrugs with the *in situ* rat brain perfusion technique. These prodrugs were D-glucose-ketoprofen ester and D-glucose-indomethacin ester. Both tested prodrugs demonstrated reversible concentration dependent inhibition of brain uptake of the radiotracer [^{14}C]D-glucose in the *in situ* rat brain perfusion model, indicating that the prodrugs bind to the GluT1. Both prodrugs were able to cross the BBB and gain entry into the brain tissue. In addition, the uptake of the prodrugs was decreased significantly when 5 °C perfusion medium was used,

indicating that the uptake was carrier-mediated. Furthermore, the high polar surface areas of the prodrugs indicate that passive diffusion across the BBB is limited. The uptake of the ketoprofen-glucose prodrug was decreased when 50 mM concentration of D-glucose was added to the perfusion medium. In addition, the uptake of ketoprofen-glucose prodrug did not become saturated even at 15 mM concentration, which suggests that the affinity of the prodrug for GluT1 is low and the capacity of the transporter is high. It is also possible that the passive diffusion of ketoprofen-glucose prodrug is significant enough to obscure the saturation of GluT1-mediated uptake. In the case of indomethacin-glucose prodrug, the uptake did not decrease when 50 mM D-glucose was added into the perfusion medium. However, the unspecific inhibition of GluT1 using lowered temperature suggests that the uptake of the prodrug is, at least, partly GluT1-mediated. The lack of inhibition of the prodrug brain uptake by 50 mM D-glucose indicates that the affinity of the indomethacin-glucose prodrug for the transporter is much higher than the affinity of D-glucose or ketoprofen prodrug. This hypothesis is further supported by the low IC50 value of indomethacin-glucose prodrug. These results strongly suggest that a hydrophilic drug can be attached to the hydroxyl group of D-glucose at the carbon 6-position and still maintain the affinity of the D-glucose promoiety for the GluT1 transporter. However, glucose as a promoiety has several limitations. The structure of glucose limits the amount of drug molecules to which biodegradable bonds can be linked. In essence, only drugs that bear a carboxyl acid group can be linked without a linker/spacer with glucose. In addition, the stability of ester prodrugs in the systemic circulation is not adequate for clinical use if the target of the drugs resides in the brain. Therefore this prodrug technology demands further development before being applicable to *in vivo* drug delivery.

8.3 CONCLUSIONS

It is concluded that:

1. It was possible to modify and combine *in vitro*, *in situ* and *in vivo* methods, in order to evaluate the mechanism of brain uptake, and the distribution in the brain parenchyma of developed amino acid and glucose prodrugs.
2. L-Tyrosine can be conjugated with drug a molecule with biodegradable linkage to form a prodrug that is able to cross the BBB via LAT1. However, the ester bond between ketoprofen and L-tyrosine is too labile *in vivo*.
3. Other amino acids than LAT1 substrates such as L-lysine can be used to form prodrugs that are LAT1 substrates. This enables the use of different prodrug bonds between the parent drug and the amino acid.
4. Amino acid prodrugs can deliver the parent drug across the BBB and further into the brain ICF *in vivo*.
5. D-Glucose can be conjugated with drug molecule with biodegradable linkage to form a prodrug that is able to cross the BBB via GluT1. However, the ester bond between the parent drugs and D-glucose is too labile *in vivo*.

Mikko Gynther

Blood-Brain Barrier Transporters in CNS Drug Deliery

Design and Biological Evaluation of LAT1 and GluT1 –Targeted Prodrugs

The aim of this doctoral thesis was to evaluate the possibility to utilize transporters present at the blood-brain barrier for enhanced brain uptake of drugs. The study is divided into three parts. In the first two parts, the ability of LAT1 to transport amino acid prodrugs across the blood-brain barrier is evaluated. The third part describes the evaluation GluT1 mediated brain uptake of glucose prodrugs.

UNIVERSITY OF EASTERN FINLAND

Publications of the University of Eastern Finland
Dissertations in Health Sciences

ISBN 978-952-61-0212-2