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KLONIRANJE, IZRAŽANJE IN FARMAKOLOŠKA KARAKTERZACIJA NUKLEOBAZNIH TRANSPORTERSKIH IN RECEPTORSKIH PROTEINOV

CLONING, EXPRESSION AND PHARMACOLOGICAL CHARACTERIZATION OF NUCLEOBASE TRANSPORTERS AND RECEPTORS

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJE

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STATEMENT

I hereby declare that this Master thesis was done by me under supervision of Dr. Meryem Köse, Dr. Anke C Schiedel, Prof. Dr. Borut Štrukelj and Prof. Dr. Christa E. Müller.

Student's signature: _____

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ABSTRACT

Nucleobase transporters and receptors facilitate the movement of nucleosides and nucleobases across plasma membranes and are commonly targeted in the treatment of cancers, viral infections, heart diseases and other. Therefore, they still remain one of the most studied group.

This master thesis discusses the characterisation of the human Equilibrative Nucleoside Transporters (ENTs) and the mouse Mas-related G-protein coupled receptors subgroup A (mMrgA). The main objective of the research was to develop *in vitro* pharmacological assays for screening of the potential active molecules.

Firstly, the DNA of respective genes was obtained and subcloned into new expression vector. Then, the cell line stably expressing gene of interest was generated in β-arrestin Chinese Hamster Ovary cells by lipofection and selection with antibiotic G418.

Parts of human Equilibrative Nucleoside Transporter 1 (hENT1) and human Equilibrative Nucleoside Transporter 2 (hENT2) were isolated from Human Embryonic Kidney 293 cells. The *in vitro* uptake assay was developed on 1321N1 astrocytoma cells with [^{3}H] adenine (30 nM) as transported species. Adenine was transported in a time-dependent manner reaching plateau after 40 minutes. The half maximal inhibitory concentration (IC$_{50}$) of dipyridamole was 0.496 ± 0.082 nM.

The mouse Mas-related G-protein coupled receptor member A1, A2b and A6 cDNA were isolated and cloned. The mMrgA6 was subcloned into expression vectors and stably expressed in β-arrestin Chinese Hamster Ovary cells. The success of cell transfection was evaluated by cyclic adenosine monophosphate (cAMP) measurements assuming that mMrgA6 signals went through G$_i$ pathways, using adenine as proposed agonist. cAMP level was decreased, however only for 7.75 pmol (31.0 %). The calculated half maximal effective concentration (EC$_{50}$) was 977 nM.

We have successfully developed an *in vitro* adenine uptake and cAMP assays, which could be used for screening of potential active substances; however further improvements of both assays are desired.

Key words: human Equilibrative Nucleoside Transporter (hENT), mouse Mas-related G-protein coupled receptor member A (mMrgA), *in vitro* pharmacological assays

EXTENDED ABSTRACT IN SLOVENE LANGUAGE
(RAZŠIRJEN POVZETEK V SLOVENSKEM JEZIKU)

Prenašalni in receptorski proteini nukleobaz so pomembni za prenos nukleozidnih in nukleobaznih učinkovin preko plazemskih membran in membran organelov ter so mnogokrat tarče učinkovin, ki se uporabljajo za zdravljenje raka, virusnih okužb, srčnih boleznih in drugih. Preko 40 % učinkovin na trgu deluje tarčno na nukleobazne prenašalne in receptorske proteine, prav zato le-ti ostajajo najbolj preiskovana skupina zaradi majhnega nivoja izražanja v specifičnih celicah, kar je pomembna lastnost, ki jo izkoriščamo pri oblikovanju učinkovin s tarčnim delovanjem.

Magistrska naloga je razdeljena na dva dela: (1) karakterizacija človeških nukleozidnih prenašalnih proteinov tipa 1 in 2, ki omogočajo dvosmerni prenos nukleozidnih in nukloebaznih učinkovin in (2) karakterizacija mišjih receptorjev, ki sodijo v skupino receptorjev sklopljenih s proteinom G.

Nukleozidni prenašalni proteini se nahajajo na površini celic mnogih tkiv v človeškem telesu in sodelujejo pri prenosu purinskih in pirimidinskih nukleozidov ter nukleobaz v celico. Trenutno je razumevanje njihove fiziološke in patofiziološke vloge pomanjkljivo, a raziskovalni članki predpostavljajo, da bi inhibitorji prenašalcev nukleozidov lahko igrali pomembno vlogo pri obvladovanju kronične bolečine. Med znane močne inhibitorje nukleozidnih prenašalcev štejemo nitrobenzil-merkaptopurin ribonukleozid in koronarne vazodilatorje (npr. dipiridamol in dilazepam), med srednje močne oziroma šibke pa tirozin- in serin-/ treonin- kinazne inhibitorje ter benzodiazepine.

Podobno tudi za receptorje, sorodne z receptorji sklopljenimi s proteinom G, predpostavljajo, da so vpleteni v nociceptivno in nevropatsko bolečino, vendar njihova fiziološka in patofiziološka vloga prav tako ostaja v veliki meri neznanka, kot tudi nativni ligandi, agonisti in antagonisti. Že več let raziskovalci poskušajo razložiti veliko pojavnost teh receptorjev pri glodavcih (50 različnih podtipov v mišjih in 11 podtipov v podganah) in manjšo pojavnost pri človeku (trenutno določeni le 4 podtipi).

Glavni namen te magistrske naloge je razvoj *in vitro* farmakoloških testov, ki bodo omogočili nadaljnjo farmakološko karakterizacijo prenašalnih in receptorskih proteinov nukleobaz. Naš cilj je bil torej pridobiti DNA omenjenih prenašalcev in receptorjev, in jih izraziti v novih vektorjih ter generirati nove celične linije s stalno izraženimi prenašalnimi in receptorskimi proteini, ki bodo omogočale njihovo karakterizacijo.

DNA transportnih proteinov smo pridobili z izolacijo mRNA iz človeške embrionalne celične linije (HEK 293), ki smo jo nato prepisali v DNA in z verižno reakcijo s polimerazo (PCR) dogradili komplementarno verigo. Omenjeno zaporedje smo nato vstavili v pJET vektor, transformirali kemokompetentno bakterijo ter izolirali in preverili ustreznost nukleotidnega zaporedja. Ugotovili smo, da smo uspeli izolirati le določena dela nukleozidnih prenašalcev podtipa 1 in 2.

Razvoj *in vitro* farmakološkega testa smo nadaljevali na celicah astrocitoma, ki vsebujejo nukleozidne prenašalce. Ugotovili smo, da se privzem adenina v celice s časom povečuje in doseže plato po približno 40 minutah. Za inhibitor dipiridamol smo določili IC_{50} vrednost 0.496 ± 0.082 nM (Slika 1).

Privzem [^{3}H] adenina v 1321N1 astrocitomske celice

$IC_{50} = \mathbf{0.496} \pm 0.082$ nM (n=6)
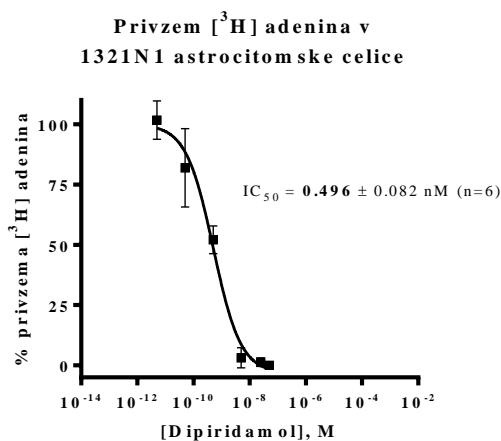
Slika 1: Graf prikazuje privzem adenina v celice v odvisnosti od koncentracije inhibitorja
Pred začetkom 2-minutnega privzema adenina v celice, smo celice inkubirali z naraščajočimi koncentracijami inhibitorja (dipirdamola). Graf prikazuje krivuljo odstotka privzema adenina v celice v odvisnosti od koncentracije dipiridamola.

DNA receptorje, sorodne receptorje sklopljene s proteinom G, smo že kupili v vektorjih, s katerimi smo transformirali kemokompetentne bakterije in DNA nato izolirali s kompletom ZymoResearch Plasmid Miniprep Classic ter preverili ustreznost nukleotidnega zaporedja. Pri podtipu 1 smo imeli 8 točkovnih mutacij, ki smo jih odstranili s postopkom usmerjene mutageneze.

Nukleotidno zaporedje se je popolnoma ujemalo pri podtipu 6, zato smo le-tega subklonirali v Prolink vektorja, ki imata sposobnost izražanja v sesalskih celicah. Stabilno celično linijo smo generirali na ovarijskih celicah kitajskega hrčka z lipofekcijo z Lipofektaminom 2000 (*Life Technology, Nemčija*), čemur je sledila selekcija z antibiotikom G418.

Uspešnost generiranja stabilne celične linije, ki izraža receptor, soroden z receptorjem sklopljenim z proteinom G, podtip 6 smo preverili z *in vitro* testom, pri katerem določimo koncentracijo cikličnega amino monofostafata (cAMP). *In vitro* test smo izvedli pri različnih koncentracijah adenina in predpostavili primarno pot sporočanja receptorja G_i.

V p l i v   a d e n i n   n a   m M r g A 6

(h o m o g e n a   e k s p r e s i j a   r e c e p t o r j a   v   β - a r e s t i n   o v a r i j s k i h   c e l i c a h   k i t a j s k e g a   h r c k a)
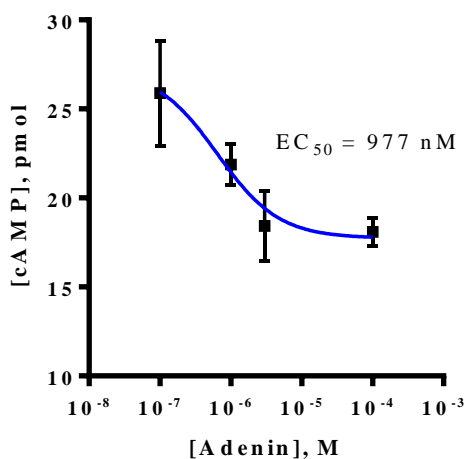
Slika 2: Graf prikazuje zmanjšanje nivoja cAMP pri višjih koncentracijah adenina

Rezultati pokažejo znižanje nivoja cAMP ob večjih koncentracijah adenina, vendar se nivo cAMP zniža zgolj za 7,75 pmol (31,0 %). Vrednost izračune polovične maksimalne efektivne koncentracije adenina je 977 nM. Potrebne so nadaljnje raziskave, da bomo lahko z gotovostjo trdili, da je adenin agonist.

Zaključimo, da sta razvita *in vitro* farmakološka testa primerna za karakterizacijo neznanih prenašalcev in receptorjev ter za iskanje novih aktivnih učinkovin, kljub temu pa bi bile nadaljnje izboljšave priporočljive.

Ključne besede: nukleozidni transporterski in receptorski proteini, Receptor sklopljen s proteinom G, *in vitro* farmakološki test

LIST OF ABBREVIATIONS

ARMS1,	pCMV-ARMS1-PK2
ARMS2	pCMV-ARMS2-PK2
BLAST	Basic Local Alignment Search Tool
bp	base pair
cAMP	Cyclic adenosine monophosphate
CHO	Chinese Hamster Ovary cells
CK2	Casein kinase 2
CNT	Concentrative Nucleoside Transporter
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
EC_{50}	Half maximal effective concentration
ECL	Extracellular loop
ei	equilibrative, insensitive
ENT	Equilibrative Nucleoside Transporter
es	equilibrative, sensitive
FCS	Fetal calf serum
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinases
GTP	Guanosine triphosphate
G_{α}	α subunit of the G-protein
$G_{\beta\gamma}$	β and γ subunit of the G-protein
h	human
HEK	Human Embryonic Kidney
IC_{50}	Half maximal inhibitory concentration
IL	Intracellular loop
IP3	Inositrol-1,4,5-triphosphate
K_{m}	The Michaelis constant in Michaelis–Menten kinetics
LB medium	Luria-Bertam medium
m	mouse
MidiPrep	midi-scale DNA preparation
MiniPrep	mini-scale DNA preparation
NBMPR	Nitrobenzylmercaptopurine ribonucleoside or nitrobenzylthioinosine
NT	Nucleoside Transporter
OD	Optical density
PCR	Polymerase chain reaction
PKC	Protein kinase C (PKC)
r	rat
RT	Room temperature
SAR	Structure–activity relationship
SLC	Solute Carrier Transporter
SNSR	Sensory neuron-specific receptor
TMH	Transmembrane helix
v_{max}	maximum rate

1 INTRODUCTION

1.1 NUCLEOSIDE TRANSPORTER FAMILY

Nucleosides and nucleobases have diverse pharmacological applications. They are precursors of nucleic acids and also modulators of cells homeostasis of important signalling molecules that modulate neurological and cerebrovascular functions. Clinically, cancer, viral infections and heart diseases are commonly treated by nucleoside and nucleobase drugs (1,2).

Due to the hydrophilic nature of pyrimidine and purine nucleosides their movement across plasma membranes and some organellar membranes is facilitated by specialized nucleoside transporters, which belong to the superfamily of solute carrier (SLC) transporters. In mammalian cells, there are two types of nucleoside transporters: the SLC28 family of Concentrative Nucleoside Transporters (CNTs), driven by a transmembrane cation (Na^+) gradient, and the SLC29 family of the Equilibrative Nucleoside Transporters (ENTs), bidirectional transporters or Na^+-independent transporters. Six CNTs (CNT1-6) and four ENTs (ENT1-4) are encoded in mammalian cells to the present date (3,4). The transporters differ in their permeant selectivity, sensitivity to inhibitors and distributions in tissues (5).

1.1.1 EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS

The Equilibrative Nucleoside Transporters (ENTs) are cell surface proteins widely distributed in mammalian tissues. Interestingly, each cell usually possesses more than one of the four ENT subtypes, whereas their abundance in tissues varies. This suggests their high regulation and specific localization in different cells and tissues (4,6). At the moment it is not reported that ENTs participate in pathogenesis of human disease; however studies in mouse suggested a role of ENT inhibitors in treatment of chronic pain in addition to a pharmacological application mentioned in chapter 1.1 (6).

In 1996, the cDNA of the human ENT1 was purified from erythrocytes for the first time. The cloning of the hENT2 followed next year due to high identity with hENT1 sequence. The ENT1 and 2 were also studied on rats and mice and are the best characterised members of the family. hENT3 and 4 were only cloned recently as a result of the human genome project and their characteristics remain largely unknown (6).

During the discovery, the classification based on sensitivity to NBMPR was very important: 1) *es* (equilibrative, sensitive) group, in which ENT1 and 3 belong to, is sensitive to NBMPR,

however the sensitivity of ENT1 is significantly higher than the sensitivity of ENT3, and 2) *ei* (equilibrative, insensitive) group, in which ENT2 and 4 belong to, is insensitive (3).

NBMPR is characterised as a classical inhibitor of ENT transporters, as well as coronary vasodilatators (e.g. dipyridamole and dilazep). Dipyridamole and dilazep bind on the permeant-binding site resulting in inhibition of hENT1 and 2. Apart from that, less potent inhibitors, such as tyrosine- and serine/threonine-kinase inhibitors and benzodiazepines, are also known to inhibit ENTs (3).

The characterisation of ENTs continues with determination of K_m and V_{max} values for substrates. In general, the ENT1-3 subtypes transport similar purine and pyrimidine nucleosides, in addition ENT2 transports also nucleobases (Figure 1). Adenosine is the only proven substrate of ENT4 (4).
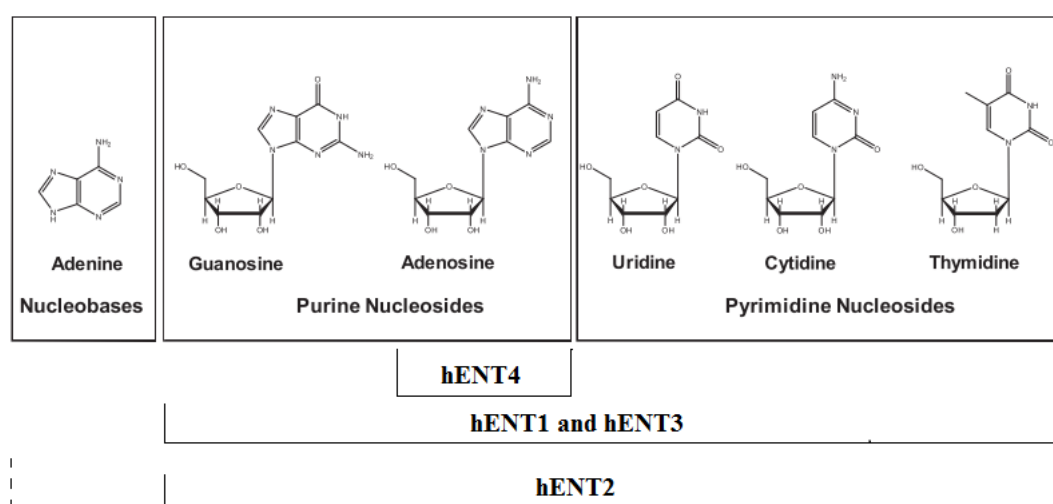
Figure 1: The ENTs' substrate review

At present no ENT crystal structure are known. However, the topology model (Figure 2) made by bioinformatics tools, suggests that ENT consists of 11 transmembrane helices (TMHs) with intracellular *N*-terminus and an extracellular *C*-terminus, has been experimentally confirmed. The topology experiments furthermore suggest one *N*-glycosylation site at hENT1 on extracellular loop1 (ECL1) connecting TM1 and 2 playing crucial role in binding NBMPR, whereas hENT2 has two *N*-glycosylation sites on ECL1 necessary for the correct targeting of the transporter to the plasma membrane. The residues in TM3-6 determine sensitivity or resistance to NBMPR and coronary vasodilator inhibitors, whereas residues in TM5-6 impact nucleobase transport. Studies also suggest that TMHs 1, 2, 4, 5, 8 and 11 cluster around the solvent-accessible permeant binding site, in other words

they are involved in the formation of the translocation channel (1). The hENT1 and 2 transporters also possess protein kinase C (PKC) and casein kinase II (CK2) phosphorylation sites in the intracellular loop 1 (ICL1) or ICL3 (4).
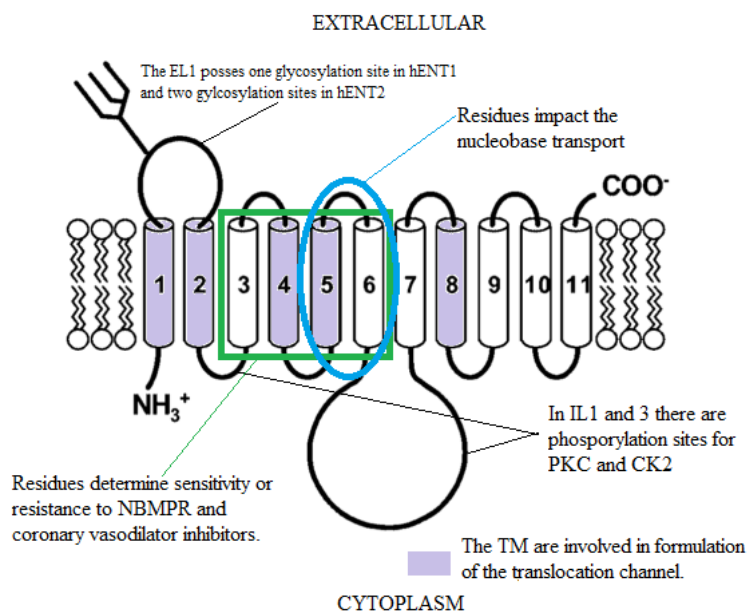
Figure 2: Predicted topology model of ENT based on bioinformatics approaches
The figure suggests the 11 transmembrane helices proposed topology. The important residues are marked and explained. Figure is adapted from Casado, n.d.

1.1.1.1 Equilibrative Nucleoside Transporter 1

The human ENT1 (hENT1) is a 456 amino acids long protein, which has 78 % identical residues with rat homologue and 79 % identical residues with mouse protein. It is ubiquitously distributed in mammalian tissues, abundant in human brains (frontal and parietal lobes of the cortex), kidney cortex (on the basolateral surface of tubular epithelial cells) and situated in the plasma membrane (6).

The important residues are summarised in Figure 2, however hENT1 lacks a potential casein kinase II phosphorylation site (6).

It transports a wide range of purine and pyrimidine nucleosides with K_m values in the range of 50-680 μM. It is suggested that ENT1 has an important role in the regulation of adenosine levels in brain. Antiviral nucleosides are poorly transported and the nucleobase uracil is not transported. Interestingly, rat ENT1 is not inhibited by dipyridamole or dilazep (4,6).

1.1.1.2 Equilibrative Nucleoside Transporter 2

The human ENT2 (hENT2), a 456-residue protein, is 46 % identical to hENT1, 88 % identical to rat and mouse ortholog. As ENT1 it is also distributed in a wide range of tissues,

such as brain, heart, placenta, thymus, pancreas, prostate and kidney. The studies suggest its important role in uptake and efflux of adenosine metabolites during exercises and recovery due to its high abundance in skeletal muscle (3,6).

As mentioned previously, apart from a wide range of purine and pyrimidine nucleosides also nucleobases (except cytosine) can be transported by hENT2. Interestingly, it has also been proven that hENT2 is able to transport a protein. On the other hand its affinity for adenosine is almost three-times lower compared to hENT1. Furthermore, the hENT2 is a good transporter of drugs used in HIV therapy (e.g. azidodeoxythymidine, dideoxycytidine and dideoxyinosine ddI). On the other hand NBMPR, dipyridamole or dilazep only weekly inhibit hENT2 (4,6).

Unfortunately, a lot is still unknown about physiology and pharmacology of ENT2 since its distribution is very similar to ENT1 distribution, but the activity is lower (3).

1.1.1.3 Equilibrative Nucleoside Transporters 3 and 4

The human ENT3 (hENT3) is a 475-residue protein with 29 % identity in sequence to hENT1. The hENT3 differs from hENT1/2 in a very long (51 residues) hydrophilic *N*-terminal region preceding TM1. Due to hydrophilic *N*-terminus it is possible that hENT3 reside predominantly in an intracellular compartment rather than at the cell surface. It is widely expressed in human tissues, in particular in placenta (6).

The human ENT4 (hENT4) is a 530-residue protein and is 86 % identical to its mouse homologue. Interestingly, the *Drosophila melanogaster* gene and the *Anopheles gambiae* gene are closely related to hENT4 proteins. However recent characterisations of the ENT4 cDNA confirmed that these proteins transport nucleosides (6).

1.2   G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors (GPCRs) compose the largest group of receptors in human. The receptors are very diverse in structure and function. They respond to different endogenous and exogenous ligands. They are represented in almost all types of tissues; however they are usually expressed at low levels and in specific cell types. These characteristics make them perfect drug targets. More than 40 % of approved drugs on the market target GPCRs. Additionally, there are many receptors among the GPCRs that still have unknown structure, function and endogenous ligands, so they remain most studied gene family (7,8).

Recently, a new classification system was presented called GRAFS. Classification is based on phylogenetic analysis and one of its main advantage is that this system includes the majority of GPCRs in the human genome despite the endogenous ligand is known or not. There are five main families: Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F) and Secretin (S). Schiöth and Fredriksson claim that the GRAFS classification is very useful for the classification of GPCR in different species as well (9).

GPCRs are cell surface molecules, which transduce extracellular signals into intracellular pathways. They form seven stretches of 25-35 consecutive amino acid residues that due to their hydrophobicity form alpha helixes or so called transmembrane helixes (TMHs). TMHs are connected by three extracellular loops (ECL) and three intracellular loops (ICL), where the *N*-terminus end is on the extracellular side of the membrane and the *C*-terminus is in the cytoplasm (8,9).

The canonical model of GPCR signalling defined by four different G-protein classes presented by Gilman is generally accepted ($G_{i/o}$, G_s, $G_{q/11}$, $G_{12/13}$). Signalling starts when active receptor is phosphorylated by GPCR kinase (GRK). This induces receptor converting into a target with high affinity binding of arrestin, which leads in binding arrestin on surface of receptor and heterotrimeric G-protein complex binding. Conformational change of the receptor is induced enabling the receptor to catalyse the exchange of GDP and GTP on the α-subunit of the G-protein (G_α). Dissociated G_α and $G_{\beta\gamma}$ active form from G-protein effect levels of cAMP (G_s – increase cAMP level, G_i – decrease cAMP level), diacylglycerol (G_q – increase DAG level), inositrol-1,4,5-triphosphate (G_q – increase IP_3 level) and Ca^{2+} (G_q – increase Ca^{2+} level) or activate RhoGEF ($G_{12/13}$) (10,11).

In addition, GPCR signalling is also influenced by arrestins, homo- and hetero-dimerization of individual, monomeric GPCR molecules (10).

1.2.1 MAS-RELATED GENES

The Mas-related G-protein coupled receptors (Mrg) receptors are a large subfamily of GPCRs, which are clustered within the rhodopsin clade of GPCRs (12). They are also called sensory neuron-specific receptors (SNSRs) and are situated in the specific set of dorsal root ganglia suggesting involvement in the sensation or modulation of nociceptive and neuropathic pain; however, their physiological functions remain mostly unknown and the same is true for their ligands (13).

Alignment analysis show only 45-65 % homology between human Mrg (hMrg) sequence and mouse Mrg (mMrg) or rat Mrg (rMrg) sequence, resulting in difficulties finding human orthologous pairs (13). Therefore, researchers continue to question the extensive sequence diversity of Mrgs in rodents (e.g. 50 different sequences in mice and 13 different sequences in rats) in comparison to lower sequence diversity in humans. To the present date only 4 Mrg receptors were encoded in humans. In rodents sequences can be divided into 4 subgroups: MrgA, MrgB, MrgC and MrgD, whereas it is interesting that in rats there is only one member in each subgroup. On the other hand, all subfamilies have many members in mice. There are two exceptions: there is only one MrgD member in mice (mMrgD) and there are ten members of rat MrgB (rMrgB), which means that in between mice and rats there are differences in subfamilies MrgA and MrgC. Even though these observations do not explain genomic expansion events that might be an atypical feature of mice, they show reduction in the complexity of Mrg diversity in rodents to a number of discovered sequences in humans (14).

Several ligands have been identified for these receptors and most of them have been peptides, which possess anti-nociceptive properties. On the other hand, also great diversity in efficacy and structure activity relationships for ligand/receptor interactions is observed. Moreover, none of these are high-affinity agonists. This suggests that ligand-binding mechanisms of Mrg receptors are different from typical GPCRs (13,15). Moreover, there is also functional diversity amongst Mrg receptors suggested due to the differences in ligand specificity (16).

The structure of Mrg receptors is highly conserved, which suggests that receptors share their functions. They consists of 7 TMH with extracellular N-terminus and intracellular C-

terminus. Interestingly, the N-terminus is short, only 3-21 residues and no signal peptide (17).

1.2.1.1 MrgA subgroup

The MrgA receptors subgroup is the biggest group within the Mrg subfamily. They have already been discovered in mouse, rat and rhesus monkey, however not in humans (12). MrgA subgroup in mice has higher sequence diversity as in other species, however analysis performed by Dong et al. indicates a low possibility that this is result of positive selection, so up to now this event has not been explained (17).

Since rodent bioassay testing models are generally used for medicine screening, there is an important need for human orphans in rodents. In human, four types of the MrgX subfamily were sequenced and characterised. Despite high Mrg diversity in mice, orphans for hMrgX receptors were not found in mice. However the phylogenetic analysis, in which Mrg gene sequences were aligned, show the greatest similarity between hMrgX and mMrgA subgroups. Studies furthermore suggested that mMrgA receptors perform similar functions as the hMrgX receptors (18).

For most of the MrgA receptors ligands are not known yet. Heo et al. has predicted binding sites for adenine and adenine was also suggested to be the ligand for the MrgA subgroup (16,19). In addition, adenine was proven to be a ligand for rMrgA. However it is still not proven that adenine is a ligand for other receptors in the MrgA subgroup. Bender et al. has also tested mMrgA2 and adenine was not found as ligand. mMrgA1 and mMrgA4 were activated by RFamide peptides, but peptides might not be endogenous ligands of those receptors. Until now, it has not been proven that adenine is the ligand of any of the human Mrg receptors (20).

In my research work, I have focused on cloning, sequencing and characterisation of three genes of mMrgA subgroup, in particular mMrgA1, mMrgA2B and mMrgA6.

The mouse **MrgA1** is a 304 residues long protein coupled to the $G\alpha_{q/11}$, but not to $G\alpha_{i/o}$ or $G\alpha_s$ signalling pathways meaning that its specific agonists stimulate dose-dependent increase in intracellular free Ca^{2+}, but do not affect basal or forskolin-stimulated levels of intracellular cAMP. Peptides with common C-terminal –RF(Y)G or –RF(Y) amide are suggested as endogenous ligands, however there was no response shown for adenine (16).

The mouse **MrgA2b** is also consisted of 305 amino acids and the mouse **MrgA6** is 301 residues long. The endogenous ligands of both receptors are still unknown.

1.3 PHARMACOLOGICAL ASSAYS

The pharmacological *in vitr*o assays have an important role in drug discovery, especially in studying interactions between receptors and ligands or transporters and molecules. There are many advantages, such as lower costs, no ethical dilemma and relatively simple establishment in comparison to *in vivo* animal studies. Cell-based assays can enable high-throughput screening of chemical libraries and have also suitable precision to define the structure-activity relationship (SAR). Therefore nowadays these assays are used in pharmacology, biochemistry and cell biology on a daily basis (21).

The *in vitro* assays can be used for characterization of agonists, inverse agonists, antagonists, and inhibitors (22).

The establishment and development phases of a new assay are very important for valid and significant results. Generally, we can divide each pharmacological assay in three parts: cells or tissue preparation, incubation and measurement. Firstly, the best expression system needs to be considered and then preparation conditions are optimised. Various cell lines are very often transfected with the cDNA of interest. During incubation there is an interaction between small molecule and protein, however this part can vary a lot between different assays. There are also a variety of labelling and detecting techniques available, such as radioactive method, fluorimetry, luminescence, absorbance, immunoactivity, and scintillation proximity assay (23).

1.3.1 TRANSPORTER ASSAY WITH RADIOLIGAND UPTAKE

The uptake assay is widely used to investigate activity of transport for transporter proteins. There are three basic steps: preparation of cells or membranes containing the protein of interest, initiation of uptake by providing energy and/or substrate and measurement of the substrate intake. There are different expression systems available for uptake assays with their own advantages and disadvantages, for example the advantage of mammalian cells is that the transport protein is in its natural state. On the other hand living cells are able to metabolize, modify and degrade substrates. This disadvantage can be limited if the assay is performed on ice (4).

1.3.2 CYCLIC AMP RADIOLIGAND BINDING ASSAY

The radioligand binding assays are based on competition, saturation or kinetic binding.

In the cAMP competing binding assay there is a competition between unlabelled cAMP and a fixed quantity of [^{3}H] labelled compound for the binding site on the highly specific and affine binding protein, which is added to the reaction mixture. An essential part of the assay is the ability to separate the bound from the unbound molecules. Centrifugation, equilibrium dialysis or filtration are only few examples of techniques, with which we can achieve separation followed by measurement of bound and labelled cAMP. Later, the amount of unlabelled cAMP can be determined by linear standard curve (24).

2   RESEARCH AIM AND OBJECTIVES

2.1   AIM

The pharmacological *in vitro* studies are an important and necessary tool for the development of new drugs and the characterisation of new targets. The structure of many receptors, and also their pharmacological and (patho)physiological function, remain unknown. On the other hand for many diseases known therapies are not effective enough. Therefore, there is a need for drug discovery and characterisation of new therapeutical targets.

G-protein coupled receptors and nucleoside transporters are two groups of proteins with huge potential as drug targets in nociceptive pain and neurological diseases. The aim of this research work is to establish and then optimise *in vitro* assays, which will enable finding of mMrgA receptors agonist and screening of potential inhibitors of human equilibrative nucleoside transporters (hENT).

2.2   OBJECTIVES

Our objectives are divided into two projects, in which we consider molecular biological and pharmacological objectives.

Objectives for the ENT project

- Establishment of conditions for an *in vitro* uptake assay for successful screening of potential hENT inhibitors
- Isolation and cloning of the hENT1 and hENT2 transporters
- Generation of cell lines stably expressing hENT1 and hENT2 transporters

Objectives for the mMrgA project

- Cloning of mMrgA1, mMrgA2B and mMrgA6
- Transient expression of cloned receptors in mammalian cell lines
- Generation of a stable cell line with mMrgA1, mMrgA2B and mMrgA6 receptors, respectively
- Establishment of cAMP binding assay and β-arrestin assay for characterisation of signalling pathways of cloned receptors
- Finding new agonist of the receptors with the established assays

3   MATERIALS AND METHODS

3.1   MATERIALS

3.1.1 EQUIPMENT

Table I: List of equipment used in the laboratory

Equipment	Manufacturer	Additional information
Agar plates	n/a	Sterile
Bacterial incubator	Innova 4200 Incubator shaker, New Brunswick	37 °C, 220 rpm
Bacterial tubes	Sarstedt	Sterile; 5 mL
Balance	n/a	0.01 - 0.1 mg
Cell culture flasks	Sarstedt	Sterile; 25 cm^2, 50 cm^2, 75 cm^2
Cell culture plates	Sarstedt	Sterile; 6-, 24-, 96- wells
Cell harvester	Brandel, Unterföhring, Germany	With GF/B glass fiber filter
Cell incubator	HERAcell® 240, Heraeus	
Cell's freezing container	Nalgene Mr. Frosty Cryo 1 °C Freezing Container	
Centrifuge	Hettich	
Cryovials	Sarstedt	Sterile
Electrophoresis chamber	n/a	
Falcon tubes	Sarstedt	25 mL, 50 mL
Freezer	n/a	-20 °C and -80 °C
Fridge	n/a	2-8 °C
Gel documentation	Universal Hood II Geldoc, Biorad	
Glass flask	n/a	Sterile
Laminar flow benches	NUNC® Safe flow 1.2, NUNC® Bioflow	
Microscope	Zeiss	
Microwave	Severin, Microwave 800	
Multipippete	Eppendorf	
Nanodrop	Titertek, Berthold	
Neubauer Chamber	Marienfeld Germany	
PCR thermo block	Biometra Tpersonal	
pH meter	691 pH Meter, Metrohm	
Pipette tips	Sarstedt	
Pipettes	Eppendorf	0.5-10 μL, 2-20 μL, 20-200 μL, 100-1000 μL
Reaction tubes	Sarstedt	0.5 mL, 1.5 mL, 2.0 mL
Scintillation Counter	PerkinElmer	Packard Tri Carb 2810 TR
Spectrophotometer	Beckman, DU® 530	

Sterile single packed serological pipettes	Sarstedt	Sterile single packed; 5 mL, 10 mL, 25 mL
Thermo block	Eppendorf, Thermomixer comfort	
Vortex	n/a	

3.1.2 CHEMICALS

Table II: List of chemicals used in the laboratory

Chemical	Manufacturer	Additional information
[^{3}H] adenine	Quotient Bioresearch, Cardiff, UK	36 Ci/mmol
[^{3}H] cAMP	Quotient Bioresearch, Cardiff, UK	34 Ci/mmol
2-mercaptoethanol	Sigma-Aldrich	14.3 M
Adenine	Acros Organics, Geel, Belgium	
cAMP binding protein	prepared from adrenal glands as described by Nordstedt et al. 1990	16.2 mg/ml
Dipyridamole	Sigma-Aldrich	
Fetal calf serum	Sigma-Aldrich	
Forskolin	Enzo Life Sciences, BML-CN100	
Gel Red Nucleic Acid	Biotrend	
Glycerol	Acros	
Lipofectamine 2000 reagent	Invitrogen	
Lambda DNA/EcoRI + HindIII Marker3	Fermentas	
Ro20-1724	Roche, Basel, Switzerland	
Scintillation cocktail		

3.1.3 KITS

In the molecular biology part of the laboratory work many kits (Table III) were used in order to optimise the time and efficiency of the process. The protocol of every product could be obtained from manufacturer website. The protocol consists of kit description and specifications, buffer descriptions and protocol for experiment.

Table III: List of kits used in molecular biology part of research work

Kits for isolation and purification of DNA	Kits for isolation and purification of RNA	Kits used in PCR
ZymoResearch Plasmid Miniprep Classic	Invitrogen: Preparation of RNA Samples prior RT-PCR	BioLabs Q5, PCR Kit
Invitrogen PureLink^{TM} HiPure Plasmid Filter Midiprep Kit	5 Prime Perfect Pure RNA Cell & Tissue	BioLine; Biomix PCR Kit
ZymoResearch DNA Clean & Concentration^{TM} 5	Invitrogen, SuperScript^{TM} II Reverse Transcriptase	Genaxxon, PCR kit

ZymoClean^{TM} Gel DNA Recovery Kit		Thermo Scientific CloneJet PCR Cloning Kit

3.1.4 BUFFERS AND SOLUTIONS

Buffers and solutions were prepared as it is described below. After mixing and adjusting pH (if needed) all buffers were sterilized by autoclaving and stored in the fridge unless otherwise is noted.

50x TAE (Tris-Acetate-EDTA) buffer: 242 g TRIS (2 M), 14.61 g EDTA (50 mM) and 57.1 mL acetic acid are dissolved in millipore water yielding a total volume of 1 L. Buffer is stored at room temperature and diluted 1:50 before usage in electrophoresis.

6x loading dye: 25 mg bromophenol blue is dissolved in 5 mL of millipore water and 5 mL of glycerol.

PBS buffer: 8.0 g NaCl (150 nM), 0.2 g KCl (2.5 mM), 1.3 g Na_2HPO_4 and 0.2 g KH_2PO_4 (1.5 mM) are dissolved in millipore water yielding a total volume of 1 L. The buffer is warmed to 37 °C before application to the cells.

0.05 % trypsin / 0.6 EDTA solution: 6 mL of 0.1 M EDTA stock solution are added to 1 L PBS buffer and autoclaved. 20 mL of a sterile 2.5 % trypsin solution and 750 μL of a sterile 0.5 % phenol red solution are added under laminar flow bench. The solution is warmed to 37 °C before application to the cells.

Tris Buffer 1.7 M: 20.594 g Tris (1.7 M) is dissolved in millipore water yielding a total volume of 100 mL.

Cold Buffer: 2.838 g HEPES and 8.006 g NaCl are dissolved in millipore water yielding a total volume of 100 mL. The pH is adjusted to 7.4 with 1.7 M Tris buffer (pH 7.0). The buffer is diluted 1:10 before the usage in the uptake assay.

HEPES Ringer Buffer: 3.94 g NaCl (135 mM), 0.189 g KCl (5 mM), 0.231 g NaH_2PO_4 (3.33 mM), 0.074 g Na_2HPO_4 x $2H_2O$ (0.83 mM), 0.069 g $CaCl_2$ x $2H_2O$ (1.0 mM), 0.104 g $MgCl_2$ x $6H_2O$ (1.0 mM), 0.901 g glucose (10.0 mM) and 0.601 g HEPES (5.0 mM) are dissolved in millipore water yielding at a final volume of 500 mL. The pH is adjusted to 7.4 with NaOH.

Triton X-100 (0.5 %): 2.5 mL of Triton (0.01 %) is mixed with 500 mL millipore water. The pH is adjusted to 7.4 with NaOH.

HBSS Buffer: 8 g NaCl (13 mM), 4.77 g HEPES (20 mM), 1 g glucose (5.5 mM), 0.1 g $MgSO_4$ (0.8 mM), 0.1 g $MgCl_2$ (1 mM), 0.185 g $CaCl_2$ (1.25 mM), 0.35 g $NaHCO_3$ (4.2

mM), 0.4 KCl (5.4 mM), 0.06 g KH_2PO_4 (0.44 mM) and 0.048 g Na_2HPO_4 (0.34 mM) are dissolved in millipore water yielding at a final volume of 1 L. The pH is adjusted to 7.4.

Lysis Buffer: 1.48 g EDTA (4 mM) and 100 μL of Triton (0.01 %) are dissolved in 1 L of millipore water. The pH is adjusted to 7.4.

3.1.5 BACTERIA GROWTH MEDIA

Luria-Bertam (LB) medium: 10 g tryptone, 5 g yeast extract and 10 g NaCl are dissolved in millipore water yielding at total volume of 1 L. The medium is sterilized by autoclaving before addition of antibiotics. The medium with antibiotic is stored in the fridge.

Luria-Bertam (LB) agar plates: 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar are dissolved in millipore water yielding at total volume of 1 L. The medium is sterilized by autoclaving and then cooled to 40 °C befor addition of antibiotics. After mixing the medium is poured into plates, where it hardens. The plates are stored in the fridge.

For the purpose of this research work LB media and agar plates with three different antibiotics were prepared: ampicillin (100 μg/mL), chloramphenicol (25 μg/mL) and kanamycin (50 μg/mL). All antibiotics were obtained from Calbiochem (Merck, Darmstadt, Germany),.

3.1.6 CELL CULTURE GROWTH MEDIA

Four cell culture growth media were obtained from Life Technology, Germany (Table IV). For purposes of our research work original cell culture growth media were modified by supplements according to requirements of specific cell line (Table V).

Table IV: List of cell culture growth media
They were all obtained from Life Technology, Germany

Medium name	Main components
DMEM (1x): Dulbecco's Modified Eagle Medium	(+) 4,5 g/L D-Glucose (+) L-Glutamine (+) Pyruvate
F-12 Nut Mix (Ham)	(+) L-Glutamine
DMEM/F-12 (1:1)	(+) L-Glutamine
Opti-MEM® (1x) Reduced Serum Medium	(+) L-Glutamine (+) HEPES (−) Phenol Red

Table V: List of modified cell culture media

The table summarise supplements added to the original media obtained from Life Techinology, Germany to meet the specific requirement of different cell lines

Name of modified medium	Cell lines	Original medium	Supplements
Basal medium 1	1321N1 astrocytoma cells HEK293 cells	DMEM (1x)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL
Basal medium 2	β-arrestin CHO cells	F-12 Nut Mix (Ham)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL Hygromycin B 200 µg/mL
Basal medium 3	CHO K1 cells	DMEM/ F-12 (1:1)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL
Selection medium 1	β-arrestin CHO cells transfected with mMrgA6	F-12 Nut Mix (Ham)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL Hygromycin B 200 µg/mL G418 800 µg/mL
Selection medium 2	CHO K1 cells transfected with mMrgA6 or adenine receptor	DMEM/ F-12 (1:1)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL G418 800 µg/mL
Culture medium 1	β-arrestin CHO cells transfected with mMrgA6	F-12 Nut Mix (Ham)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL Hygromycin B 200 µg/mL G418 200 µg/mL
Culture medium 2	CHO K1 cells transfected with mMrgA6 or adenine receptor	DMEM/ F-12 (1:1)	FCS 10 % Penicillin 100 U/mL Streptomycin 100 µg/mL 1 ml G418 (200 µg/mL)

3.1.7 BACTERIA AND CELL CULTURES

The bacteria and cell cultures used in the research work are listed in Table VI.

Table VI: List of bacteria and cell cultures used in research work

	Culture type	Manufacturer
Bacteria cultures	Escherichia coli, DH5α	Life Technologies, Darmstadt, Germany
	Escherichia coli, TOP10	Life Technologies, Darmstadt, Germany

Cell cultures	1321N1 astrocytoma cells	Sigma-Aldrich, München, Germany
	Human Embryonic Kidney (HEK) 293 cells	Sigma-Aldrich, München, Germany
	Chinese Hamster Ovary (CHO) K1 cells	ATCC, CCl-61
	ß-arrestin Chinese Hamster Ovary (CHO)	ATCC, CCl-61

3.1.8 ENZYMES

All enzymes including restriction endonucleases were obtained from New England BioLabs (Ipswich, MA, USA) and used with enclosed buffer (Table VII).

Table VII: List of enzymes and restriction endonucleases

	Name	Recognised sequence and cutting position	Enclosed buffer
Enzymes	Antarctic phosphatase	-	10x phosphatase buffer, Buffer 2
	T$_4$ DNA Ligase	-	10x Ligation buffer
Restriction endonucleases	BamIII-HF	5´...G̲GATCC...3´ 3´...CCTAG̲G...5´	CutSmart^{TM} Buffer
	BglII	5´...A̲GATCT...3´ 3´...TCTAG̲A...5´	Buffer 3.1
	DpnI	CH₃ 5´...GA̲TC...3´ 3´...CT̲AG...5´ CH₃	CutSmart^{TM} Buffer
	EcoRV - HF	5´...GAT̲ATC...3´ 3´...CTA̲TAG...5´	CutSmart^{TM} Buffer
	NheI-HF	5´...G̲CTAGC...3´ 3´...CGATC̲G...5´	CutSmart^{TM} Buffer
	XhoI	5´...C̲TCGAG...3´ 3´...GAGCT̲C...5´	CutSmart^{TM} Buffer

3.1.9 PRIMERS

Table VIII: List of designed and ordered primers

Primer name	Sequence (5' – 3')	Length	Tm (1 M Na$^+$)	% GC	Purpose
f-mMrgA6-ATG-BamHI r-mMrgA6-TGA-XhoI	CTGTCAGGATCCACCATGCATAGAAGTATCAGCATCAG TCACTACTCGAGTCACAGCTCTGCTTTGTTTCTTG	38 35	83 81	47 46	Cloning of mMrgA6
f-mMrgA6-ATG-NheI r-mMrgA6-wo-stop-BglII	CTGTCAGCTAGCACCATGCATAGAAGTATCAGCATCAG TGCTGCAGATCTCCAGCTCTGCTTTGTTTCTTG	38 33	83 81	47 48	Cloning of mMrgA6
f-mMrgA1-R26T r-mMrgA1-R26T	CCTGGAGGTATCAACATCACGATTCTGATCCCAAAC GTTTGGGATCAGAATCGTGATGTTGATACCTCCAGG	36	82	47	Change of amino acid R on position 26 into amino acid T
f-mMrgA1-I102T r-mMrgA1-I102T	CTCTTGTGCTTTTACACGATCATGATGGTTCTC GAGAACCATCATGATCGTGTAAAAGCACAAGAG	33	78	42	Change of amino acid I on position 102 into amino acid T
f-mMrgA1-N307T r-mMrgA1-N307T	CCTGAGACAGCCAAAATCATGGTGGAGATGTC GACATCTCCACCATGATTTTGGCTGTCTCAGG	32	81	50	Change of amino acid N on position 307 into amino acid T
f-mMrgA1-A46G r-mMrgA1-A46G	GTCGGGCTGACAGGAAATGGCATTGTGTTCTGGCTCCTGGGC GCCCAGGAGCCAGAACACAATGCCATTTCCTGTCAGCCCGAC	42	90	60	Change of amino acid A on position 46 into amino acid G
f-mMrgA1-S180N r-mMrgA1-S180N	GGGTGTCTGGCATTGAACTTCTTTACTGCTGC GCAGCAGTAAAGAAGTTCAATGCCAGACACCC	32	81	50	Change of amino acid S on position 180 into amino acid N
f-mMrgA1-R57H r-mMrgA1-R57H	CCTGGGCTTCTGTTTGCACAGGAACGCCTTCTCAG CTGAGAAGGCGTTCCTGTGCAAACAGAAGCCCAGG	35	86	57	Change of amino acid R on position 57 into amino acid H
f-mMrgA1-S203A r-mMrgA1-S203A	GCCTGGCTCTGGTGGCCAGGTTGTTCTGTGG CCACAGAACAACCTGGCCACCAGAGCCAGGC	31	86	65	Change of amino acid S on position 203 into amino acid A

f-mMrgA2B-ATG f-mMrgA2B-12-Exon2 r-mMrgA2B-TAG	ATGGGGGAAAGCAATGGT GAAG CAATGGTGAAGCATTTCTT GCCT TCAAGGCTCTGCTTTGTTA CTTG	22 23 23	71 70 70	50 48 43	Cloning of mMrgA2B
f-hENT1-10 r-hENT1-1353	AGTCACCAGCCTCAGGAC AG TCACACAATTGCCCGGAA C	20 19	64 58	60 53	Cloning of hENT1
f-hENT2-22 r-hENT2-1354	CGGGACAGCTACCACCTG TCAGAGCAGCGCCTTGAA	18 18	60 56	67 56	Cloning of hENT2
f-β-actin r-β-actin	GGTGGCTTTTAGGATGGC AAG ACTGGAACGGTGAAGGTG ACAG	21 22	64 68	52 55	For blank sample

3.1.10 PLASMIDS

Plasmids pCMV-ARMS1-PK2 (Figure 3), pcDNA3.1(+) (Figure 4) and pCMV-ARMS2-PK2 (Figure 5) and were used in the research project. Plasmid maps show name and length of a plasmid, antibiotic resistance and restriction sides of respective plasmids.

In addition, also pJET1.2 blunt vector (map not shown), which is resistant to antibiotic ampicillin, was used in few cases.
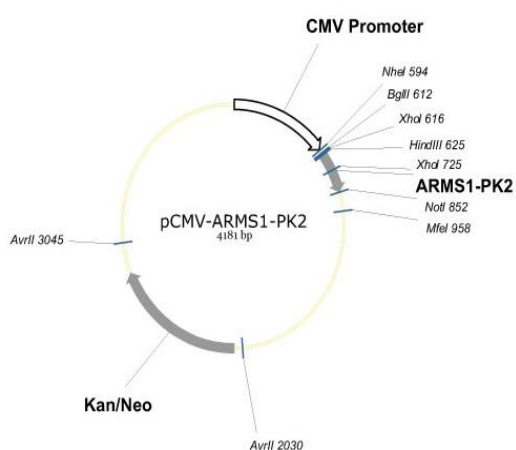
Figure 3: Plasmid map of pCMV-ARMS1-PK2 (ARMS1) vector
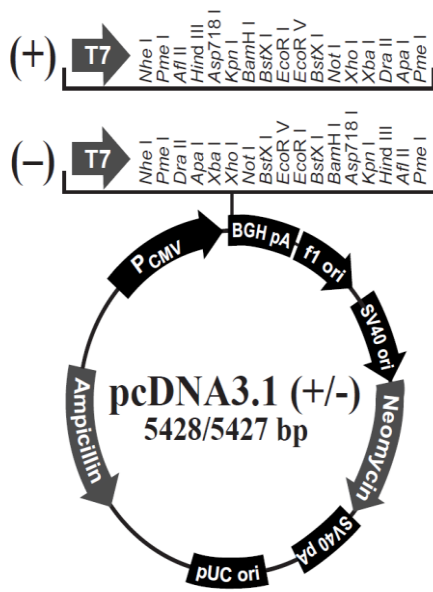
Figure 4: Plasmid map of pcDNA3.1(+/-) vector
pcDNA3.1(+) was used in the research project
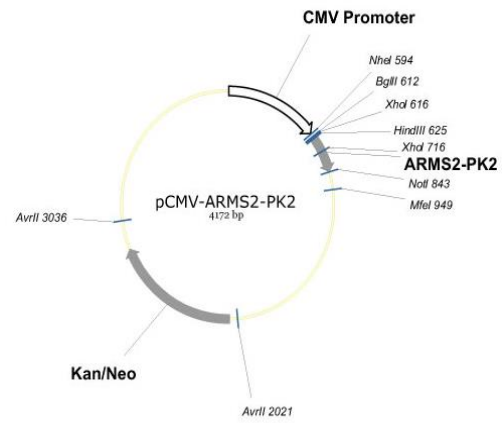
Figure 5: Plasmid map of pCMV-ARMS2-PK2 (ARMS2) vector

3.2   METHODS

3.2.1 TRANSFORMATION OF BACTERIAL COMPETENT CELLS

Transformation is a process, where exogenous DNA is inserted in bacterial cells (25). This could be done by electroporation or chemically induced competent (26). Electroporation is more expensive and more complicated method, so the second method is more used in the laboratories and it was also used in this study (27). The main purpose of transformation is the production of recombinant plasmids (28).

3.2.1.1 Production of chemocompetent cells

Production of chemocompetent cells is based on prolonged exposure in $CaCl_2$. For the efficiency of production of potent chemocompetent cells, it is important that cells are harvested, when they are in the logarithmic phase of growth (27,29).

Protocol

First 4 mL of LB medium is inoculated with 50 μL of bacterial glycerol stock of untransformed cells and bacteria are cultivated overnight at 37 °C and 220 rpm. Next morning 40 mL of LB medium is inoculated with entire 4 mL of the overnight culture and bacteria are cultivated at 37 °C and 220 rpm until the absorption of the bacterial suspension has reached an OD of 0.5 measured at 550 nm. Then the cells are pelleted at 1700 x g at 4 °C for 20 minutes. The pelleted cells are resuspended in 20 mL of sterile and cooled 0.1 M $CaCl_2$ solution and incubated on ice for 30 minutes. Incubation is followed by a second centrifugation at 1700 x g at 4 °C for 20 minutes. The cell pellet is resuspended in 2 mL of sterile and cooled 0.1 $CaCl_2$ suspension and 0.5 mL of sterile glycerol is added. 100 μL of bacterial suspension is aliquoted in 1.5 ml microtube and stored at -80 °C. After first addition of $CaCl_2$ it is necessary to work on ice all the time.

3.2.1.2 Preparation of glycerol stocks

The aim of preparation of glycerol stock is storing of bacteria containing the respective plasmid.

Protocol

1 mL of LB medium with appropriate antibiotic is added to 1 mL of overnight culture of chemo competent bacteria and incubated at 37 °C and 220 rpm for 1 hour. Then 200 µL of sterile glycerol and 800 µL of bacterial cells suspension are added in 1.5 mL microtube. Before storing at -80 °C the mixture is briefly vortexed.

3.2.1.3 Transformation of bacterial cells

In this study transformation is based on $CaCl_2$ induced cells and short heat shock (42 °C), which is followed by chilling on ice. The mechanism of uptake is still unknown, however the method is proven to be very effective (27).

Protocol

Bacterial cells of *E. coli* strand TOP10 and DH5α, described under materials (chapter 3.1.5), were used for transformation. An aliquot of bacterial suspension from the freezer is thawed on ice. 10-50 ng of plasmid is added. The suspension is mixed carefully, incubated on ice for 30 minutes, heated at 42 °C for 30 second and cooled again on ice for 2 minutes. 100 µL of LB medium is added. The new suspension is incubated at 37 °C and 220 rpm for 1 hour and then spread on LB agar plate containing the appropriate antibiotic. The inverted plates are incubated at 37 °C overnight.

3.2.2 ISOLATION AND PURIFICATION OF PLASMID DNA

There are many different methods for isolation and purification of DNA. Nowadays there are many manufacturer that offer kits, which are rapid and efficient.

3.2.2.1 Mini-scale preparation of DNA

Preparation of DNA in small scale or so called mini-scale preparation (MiniPrep) is usually done for large number of samples, so the most important characteristic is time. There are three main almost equally fast methods, whose main difference is in type of bacterial lysis: alkaline lysis, boiling methods and lithium mini-preparation (30).

The kit, used in this research, is based on alkaline lysis and Fast Spin column (31).

Protocol

Single clones from LB agar plates are incubated in 4 mL LB medium with the appropriate antibiotic at 37 °C and 220 rpm overnight. On the next day DNA is isolated by the kit Zymo Research Plasmid Miniprep™ – Classic following the protocol provided by manufacturer (31). Protocol was modified in the last step, where elution is done in 30 μL of PCR water.

3.2.2.2 Midi-scale preparation of DNA

Preparation of DNA in medium (MidiPrep) or maxi (MaxiPrep) scale does not differ a lot from mini-scale preparations. The methods are based on same principles; however more DNA is needed at the beginning (30). The kit, which is based on binding the negative charged phosphate of the DNA backbone directly to the anion-exchanged resin, is used in this study. Furthermore mRNA, proteins, carbohydrates and other impurities are removed with wash buffer (32).

Midi-scale preparations (MidiPrep) are prepared, when the appropriateness of mini-scale preparation is confirmed and more DNA is needed for following downstream processes.

Protocol

Single clones from LB agar plates are incubated in 4 mL LB medium with appropriate antibiotic at 37 °C and 220 rpm for approximately 8 hours. Then 100 mL of LB medium with appropriate antibiotic is inoculated with entire 4 mL of bacterial suspension and incubate at 37 °C and 220 rpm overnight. Midi-scale preparation is performed with kit Invitrogen PureLink™ HiPure Plasmid Filter Midiprep Kit following provided protocol (32).

3.2.2.3 Gel electrophoresis

Electrophoresis is one of the most widely used methods for purification of DNA based on separation between DNA, RNA and proteins or fragments of DNA. In an electrical field oligonucleic acids travel from negative charge to positive charge and separation is based on differences between intrinsic electrical charges. Normally, larger fragments move slower than smaller fragments and when marker is used the approximately length of DNA fragment can be defined. Furthermore linear DNA travels slower than supercoiled DNA with the same size, which is useful characteristic for checking successfulness of restriction digestion. Dyes

are usually added to samples before loading them on gel, because dye intercalate with DNA strands and enables visualisation of gel under UV light (33,34).

Protocol

250 mg agarose (1 %) is dissolved in 25 mL 1x TAE buffer by heating the solution in a microwave and then 2.5 µL Gel Red Nucleic Acid is added to the cooled solution. The solution is poured into a gel chamber with a comb. The hardened gel is transferred to the electrophoresis chamber and marker and samples are loaded. After approximately 20 minutes run at 100 V the results are visualised with UV light.

1 µL 6x loading dye per 5 µL DNA solution is added to prepare samples for electrophoresis.

3.2.2.4 Recovery of DNA from agarosis gel

DNA recovery from agarosis gel plays an important role, because some samples need to go under electrophoresis in order to be used in following downstream processes. In recovery a big amount of DNA can be lost, so improvements of this method are discussed in many articles (35).

Protocol

The cut bands of DNA samples are put 1.5 mL microtube and DNA is recovered by kit ZymoClean^{TM} Gel DNA Recovery Kit following provided protocol (36).

3.2.2.5 Purification of DNA

DNA can be contaminated with small DNA fragments, RNA, proteins and salts, which can disturb the following downstream processes. Generally, centrifugation and chemical extraction are the methods in use (34).

However, nowadays there are also many kits commercially available for purification, which are quick and efficient. DNA Clean and Concentration^{TM} kit work based on Zymo-Spin^{TM} Column (37).

Protocol

The DNA was purified after PCR reaction and restriction of PCR product. Zymo Research DNA Clean & Concentration^{TM} 5 kit was used. The provided protocol (37) is modified in last step since elution is done in 10 µL of PCR water.

3.2.3 ISOLATION AND PURIFICATION OF mRNA

3.2.3.1 Purification of RNA from cultured cells

Protocol

1321N1 astrocytoma and HEK293 cells are seeded (1.5×10^6 cells/ml) in monolayer in 6-well plates and incubated overnight at 37 °C, 5 % CO_2. The ENT1 and ENT2 transporters are isolated by usage of primers (see 0 chapter) and 5 Prime Perfect Pure RNA Cell & Tissue kit following the protocol 2C described in PerfectPure RNA Cell and Tissue Manual (38). Firstly, lysis solution is added directly to a plate of cells. The steps of RNA binding and wash 1, DNase treatment, wash 2 and RNA elution follow.

3.2.3.2 Treatment with DNAse

The RNA is treated with DNase in order to degrade external DNA before reverse transcription.

Protocol

The Invitrogen kit Preparation of RNA samples prior RT-PCR provided is used following enclosed protocol.

3.2.3.3 Reverse transcription

A reverse transcription is a process in which cDNA is synthesized from RNA. The cDNA strand is more stable than RNA strand, because RNase enzymes, which degrade RNA, do not need cofactors as DNase enzymes. cDNA is used as a template for PCR reaction (30).

Protocol

The Invitrogen kit Superscript$^{\text{TM}}$ II Reverse Transcriptase is used according to manufacturer protocol to transcriptase cDNA with oligo-dT and random hexamers (39). The blank sample is prepared as well.

3.2.4 RESTRICTION DIGESTION WITH ENDONUCLEASES

Restriction endonucleases recognise specific 4 to 8 bp long nucleotide sequence and they have the ability to cut only this specific target. The restriction endonucleases type II are most widely used in molecular biology, because they cut DNA at exactly definable sites and create fragments with a 5'-phosphate and a 3'-OH ends. The enzymes can cut both strands at the same site, so the ends are smooth (i.e. blunt ends), or they create overhangs. The overhangs can be ligated and this is the preferable way for molecular cloning, because later the efficiency of ligation is higher (30).

Before ligation a vector and insert are digested with endonucleases. An insert needs to be digested by two restriction enzymes, whereas a vector could be digested with one or two enzymes. In this study, the different vector is always digested by two enzymes in order to ensure higher proportion and the correct orientation of the insert (30).

Protocol

The appropriate enzymes and buffer (Chapter 3.1.9) are prepared for digestion of the insert and plasmid. Each insert and plasmid is digested by two different restriction enzymes and two overhangs are created. If both enzymes are active in the same buffer, one can perform only one digestion, otherwise one needs to do one reaction after the other with a purification step in between (Table IX). After digestion plasmids are purified by electrophoresis to remove the polylinker released from the plasmid, which can later disturb ligation process, and inserts are purified by DNA Clean & Concentration™ 5 kit.

Table IX: Presentation of mixture used for restriction digestion

Preparation of mixture	
DNA (plasmid or PCR product/insert)	$0.25 - 1$ μg
Reaction buffer	1 μL per 10 μL sample
Restriction enzyme	0.5 μL of each enzyme
PCR water	to complete until desired volume
Incubation	20 min at 37 °C

3.2.4.1 Dephosphorylation of digested vector

A digested vector usually has phosphate at 5'- end, which is useful for ligation. However phosphate could be removed by phosphatases (Table X) in order to prevent self-ligation of plasmids and enhance the ligation between vector and insert, which still possess both 5'-phosphate and can ligate with vector's DNA strand (30).

Table X: Protocol for dephosphorylation of digested plasmid

Preparation of mixture:	
digested plasmid	minimum 50 ng
NEB buffer 2	1 µL per 10 µL of sample mixture
10x phosphatase buffer	1 µL per 10 µL of sample mixture
antarctic phosphatase	1 µL
sterile water	to complete until desired volume
Incubation	25 min at 37 °C
Heat deactivation	5 min at 65 °C

3.2.5 LIGATION

The T4 DNA ligase is the most commonly used enzyme for ligation. In the respective buffer with ATP it is fast and adequate (30). The ligation is performed according to the protocol described in Table XI.

Table XI: Protocol for ligation

Preparation of mixture:	
cut plasmid	50 ng
insert	150 ng
10x ligation buffer (with 100 mM ATP)	1 µL per 10 µL of sample mixture
T4-ligase	0,5 µL
sterile water	to complete until desire volume
Incubation (sticky ends)	30 min at RT

3.2.6 POLYMERASE CHAIN REACTION

PCR is a method, which is widely used in molecular biology and has many different applications. Most commonly it is used for multiplying the amount of DNA; however it could be used also for removing mutations, synthesizing new strand etc.

PCR protocol (Table XII) consists of three main steps: denaturation, annealing and elongation step. Firstly, two strands of template DNA are separated by breaking H-bonds between both strands. This step is performed at high temperature, but for short period of time in order not to denaturate DNA completely. Then temperature is lowered to approximately 2 °C below the melting temperature of the oligonucleotide primers (Tm – 2 °C). Forward and reverse primers are annealed to the 3'- prime end of each strand. Lastly, the new strand is enzymatically built in direction 3'-5' prime end with DNA building-blocks, nucleotides (dNTP), by Taq polymerase. The elongation is performed at optimal temperature of Taq polymerase (72 °C). This protocol is repeated in cycles to produce higher amounts of DNA and concluded with longer elongation and cooling to 4 °C (30).

Table XII: PCR protocol for multiplying the amount of DNA

PCR Protocol:
98 °C for 10 s
98 °C for 10 s
60 °C for 1 min
72 °C for 1 min 20 s
72 °C for 10 min
4 °C STOP
30 cycles (2-4 steps)

3.2.6.1 Primer design

The primer design is an important step, because successfulness of PCR is depended on primers' annealing. Forward and reverse primers are always designed together and are complementary to 3' end of respective strands of DNA. It is important that primers start with 2-3 guanines (G) or cytosine (C) in order to induce strong binding at the beginning. Primers should consist out of 18-22 nucleotides in length with a G+C content of 45-60 %.

If primers are needed for cloning, it is much recommended to add restriction sides as well. On the 5' end a restriction site of chosen enzyme is added to primer sequence. It is important that restriction enzymes are chosen according to restrictions sides in desirable vector and that they will not cut the insert in between.

Primers for site-directed mutagenesis are usually longer between 28 and 34 nucleotides with the desired mismatched base in the middle. It is also important that the primers consist of 45-60 % G or C.

In the end primer characteristics should be checked. Good primers have melting temperature (T_m) between 55 and 70 °C. Nowadays, many programs are available to calculate T_m; however rule of thumb (**Equation** 1) is still very useful to give us approximately values. Primers are also checked if they form stable hairpins or stably homodimerize and heterodimerize, which they should not. They also need to be specific for target sequence. If primers confirm all requirements, they are ready for order.

Equation 1: Calculation of mealting temparature of sequence with Rule of thumb.
G represents number guanine, C number of cytosine, A number of adenine and T number of thymine

$$\mathbf{Tm}\ (°\mathbf{C}) = \mathbf{4} \times (\mathbf{G} + \mathbf{C}) + \mathbf{2} \times (\mathbf{A} + \mathbf{T})$$

3.2.6.2 PCR amplification

In this study, PCR is often used for amplifying the amount of DNA (Table XII). Many kits (chapter 3.1.3) are commercially available on the market and are used for this purpose.

3.2.6.3 Site directed mutagenesis

The single nucleotide mutations are successfully removed by site directed mutagenesis. The PCR program (Table XIII) is modified; elongation phase is prolonged and number of cycles is decreased.

Table XIII: PCR protocol for site-directed mutagenesis

Protocol:
94 °C for 4 min
94 °C for 20 s
55 °C for 30 s
72 °C for 10 min
94 °C for 4 min
4 °C STOP
21 cycles (2-4 steps)

3.2.6.4 Sequencing

GATC Biotech AG, Germany, perform all sequencing analysis. The samples are prepared according to their specifications.

3.2.7 CELL CULTURE

3.2.7.1 Cultivation of cells

All cell lines are grown in cell incubator with humidified atmosphere of 95 % air and 5 % CO_2 and at 37 °C. Cells used for assays are incubated at those conditions as well, unless otherwise stated. Cells are always handled under laminar air flow bench.

The adherent cells grow in monolayer in cell culture flasks and when they are 80-95 % confluent; they are passaged into new flask. If we want to split the cells, they need to be detached. First, the medium is removed from the flask and the cells are washed with PBS buffer in order to remove residual medium. Then 0.5 % trypsin /0.6 mM EDTA solution is added and incubated at 37 °C for 3-5 minutes. Integrin, which is crucial for attachment of cells, is degraded by the proteolytic enzyme trypsin. EDTA allows trypsin to work more efficiently by engaging metal ions. The detached cells are suspended in basal medium. Depending on the number of cells needed, a certain volume of cells is transferred into new flasks with basal medium.

All solutions used are preheated to 37 °C in order not to disturb cells.

3.2.7.2 Counting and seeding cells

The detached cells are counted and seeded in 6-, 24- or 96- well plates for performance of pharmacological assays. In order to ensure reproducibility of results, the same number of cells needs to be seeded in each well every time. Cells are therefore counted with a Neubauer Chamber according to the protocol provided by the manufacturer. Then the calculated volume of cells is added in each well yielding the final volume with basal medium.

Plates are incubated for 1 or 2 days (depending on the assay protocol) in the cell incubator.

3.2.7.3 Freezing of cells

Freezing of cells is very important in order to store cell lines for longer time. Freezing can be done in freezer or with liquid nitrogen.

Nalgene Mr. Frosty Cryo 1 °C Freezing Container is used for freezing. First detached cells from confluent 175 cm^2 cell culture flask are transferred into a falcon tube and centrifuged at 1000 x g for 5 minutes. The pellet is resuspended in 1 mL FCS with 10 % DMSO, which works as cryoprotectant. Suspension is added into tubes and tubes are placed in tube holder.

After freezing overnight at -80 °C, tube can be placed in storage boxes or transferred to liquid nitrogen.

3.2.7.4 Generation of stable cell lines by lipofection

Native cell lines could be transfected with a new protein by lipofection or viral transfection. In this study new cell lines are generated by lipofection.

Firstly 2.5×10^6 cells are seeded into a 25 cm^2 cell culture flask and medium without any antibiotics. After 3 hours incubation in cell incubator two tubes are prepared. In the first tube Lipofectamine 2000 is mixed with medium without supplements, in a second tube 10 μg of DNA is mixed with medium without supplements. The first tube is incubated at room temperature for 5 minutes before addition to the second tube. After gently mixing and 20 minutes incubation the mixture is added to the cells. After 48 hours, the cells are diluted 1:10 and seeded in growth medium. After 72 hours, the growth medium is changed with selection medium. The cells are left to grow until the selection is over.

The media details are described in chapter 3.1.6.

3.2.8 PHARMACOLOGICAL ASSAYS

3.2.8.1 The transporter uptake assay

Protocol

Time-dependent assay

Firstly, 0.65×10^5 cells are seeded in a 24-well plate and incubated for 2 days. On the day of the assay, cells are washed twice with 200 μL HEPES buffer Ringer solution. After washing 150 μL of HEPES buffer containing 1 % DMSO is added in 21 wells and then [^{3}H] adenine is added at different times (0, 30, 40, 50, 55, 57, 59 minutes) in order to ensure various time of uptake in respective wells (Figure 6).
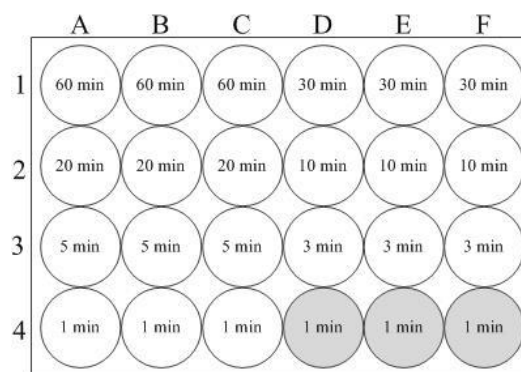
Figure 6: 24-well plate layout in time-dependent assay
The layout shows the time of uptake in the respective well. In wells coloured in grey 25 μM of dipyridamole is added.

After 1 hour the transport is stopped with 500 μL cold buffer. Cells are washed twice with cold buffer and then lysed with 0.5 % Triton X-100. Cell lysate is transferred in the scintillation vials and 2.5 mL Scintillation cocktail is added after an incubation of 9 hours

the radioactivity was measured by liquid scintillation counting (TriCarb® 2810 TR, PerkinElmer, Inc., USA) counting at an efficiency of 54 %.

Three wells are negative controls containing inhibitor. Those wells are important to determine the starting point of the curve (zero transport).

<u>Concentration dependent inhibition of adenine uptake</u>

For this assay cells are prepared at the same protocol as described above. After cells are washed twice with 200 μL HEPES buffer Ringer solution, 100 μL of HEPES buffer containing 1 % DMSO and 50 μL of inhibitor in different concentration is added to each well, except in 6 wells, where only 150 μL of HEPES buffer is added (positive control). Cells are incubated with an inhibitor for 30 minutes on ice and then a [³H] adenine is applied. The uptake is stopped by cold buffer. Cells are lysed and transferred into scintillation vials as described above. The same measurement follows.

3.2.8.2 The cAMP binding assay

cAMP is a second messenger involved in intracellular signalling pathways. As it was discussed in chapter 1, possible signalling pathways of GPCRs are the increase or decrease cAMP levels, which is converted from ATP by adenylate cyclases (40).

Screening G_s-coupled receptors is usually straightforward; whereas screening of $G_{i/o}$-coupled receptors might be more difficult. Radiometric cAMP assays has been widely used, however they are more often replaced by fluorescence or luminescence based assays now (23).

Protocol

cAMP assay was performed in two parts. Firstly, cells are seeded in 24-well plate (2.0 x 10⁵ cells/ well). After overnight incubation cells are washed twice with 300 μL 37 °C warm HBSS buffer and incubated for 2 h in 300 μL HBSS buffer at the same conditions. When incubation is finished, we proceed with the protocol for $G_{i/o}$-coupled receptors. Ro20-1724 is added in order to inhibit phosphodiesterase, which can catalyse the degradation of cAMP. After 10 minutes incubation 100 μL of agonist is added, except in wells with negative control, where only 100 μL of HBSS with 5 % DMSO is added. Agonist solutions are prepared in different concentrations, as it is shown in the Table XIV. Incubation lasts 5

minutes prior addition of 50 µL of forskolin for 15 minutes. The Forskolin is a direct activator of adenylate cyclase and increases the intracellular cAMP concentration. In the last step the supernatant is discarded with a pump and 500 µL of lysis buffer preheated at 90 °C is added. The 24-wells plates are frozen at -20 °C.

Table XIV: Preparation of adenine solutions for two 24-wells plate

Volume of 10 mM adenine stock [µL]	Volume of HBSS in 5 % DMSO [µL]	End volume [µL]	Dilution factor	Concentration of adenine solution [µM]	Final concentration of adenine in reaction wells [µM]
70	1330	1400	1:20	500	100
600	1400	2000	1:3.3	150	30
600	1200	1800	1:3	50	10
600	1400	2000	1:3.3	15	3
600	1200	1800	1:3	5	1
200	1800	2000	1:10	0.5	0.1

The second part of the assay starts with thawing the plates, which takes approximately 3 hours in the fridge. Then cells at the bottom are scratched, lysed and homogenised with the pipette. Reaction vials are prepared as follows: 3 vials determining total binding of cAMP, 3 vials determining background, 5 vials with cAMP standard solution with concentrations of 2.5, 5, 10, 20 and 40 pmol and 24 vials (if you analyse one 24-well plate) containing homogenised cell lysate (Table XV). Then 30 µL of [³H] cAMP is added to all vials and then 40 µL of cAMP binding protein is added to all vials except for the background vials. The reaction mixture is incubated on ice for 1 hour and then filtrated through a GF/B glass fiber filter with ice-cold Tris buffer using a cell harvester. In scintillation vials the filters and 2.5 mL of scintillation cocktail is added. As before prior measurement incubation takes place for 9 hours.

Table XV: Preparation of reaction vials in cAMP binding assay

The table shows the protocol for preparation of vials determining total binding of cAMP and background, vials with cAMP standards and vials containing cell lysate transferred from microtiter plate. The dilution of cAMP standards concentration of 2.5, 5, 10, 20 and 40 pmol, [^{3}H] cAMP solution and cAMP binding protein solution are prepared prior the assay.

	Total binding	Background	cAMP standards	Assay
Cell lysate	-	-	-	50 μL
Dilution of cAMP standard	-	-	50 μL	-
Lysis buffer	50 μL	90 μL	-	-
[^{3}H] cAMP (3 nM)	30 μL	30 μL	30 μL	30 μL
cAMP binding protein (8.2 mg/ mL)	40 μL	-	40 μL	40 μL
Final volume	120 μL	120 μL	120 μL	120 μL

3.2.9 DATA ANALYSIS

GraphPad Prism 6.0 was used for statistical analysis and graph presentations. At least two individual experiments were performed in triplicates prior non-linear regression analysis. Results are always shown as mean $\pm$ SEM.

4   RESULTS AND DISCUSSION

4.1   ENT PROJECT

The ENT project was planned in two parts. Firstly, a transporter uptake assay was established with 1321N1 astrocytoma and HEK293 cells. At the same time we have worked on generation of CHO cell lines stably expressing hENT1 and hENT2 transporters. The project scheme is summarised in Figure 7.
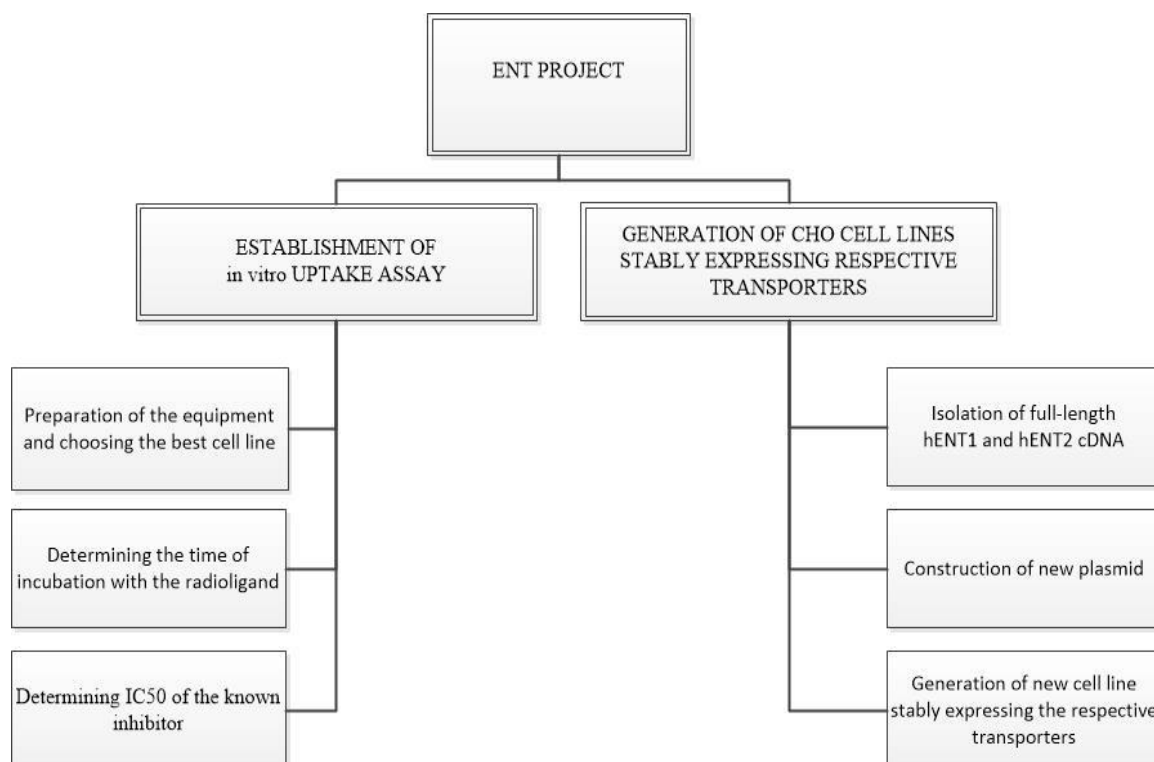
Figure 7: Research plan for ENT project

4.1.1 UPTAKE ASSAY

The objective was to establish an *in vitro* pharmacological assay for characterisation of hENTs and screening for potential inhibitors of respective transporters.

In the development process our objective was to determine two parameters: time of incubation with the radioligand and half maximal inhibitory concentration (IC_{50}) of known inhibitor; however before that it was necessary to find suitable cell lines and equipment for assay performance.

The assay was a bit more complicated from the handling perspective as usual, so therefore a special apparatus was assembled (Figure 8). The apparatus consists of three main parts: pump, waste bottle and sucking pipette. The pump ensured the vacuum and the liquid from microtiter plate was sucked in the waste bottle. In this way, the medium and buffers were removed quicker and more efficiently, so the better condition for the assay was assured. Furthermore, also our safety was higher, especially when buffer containing radioactivity was removed directly in the waste bottle (closed system).
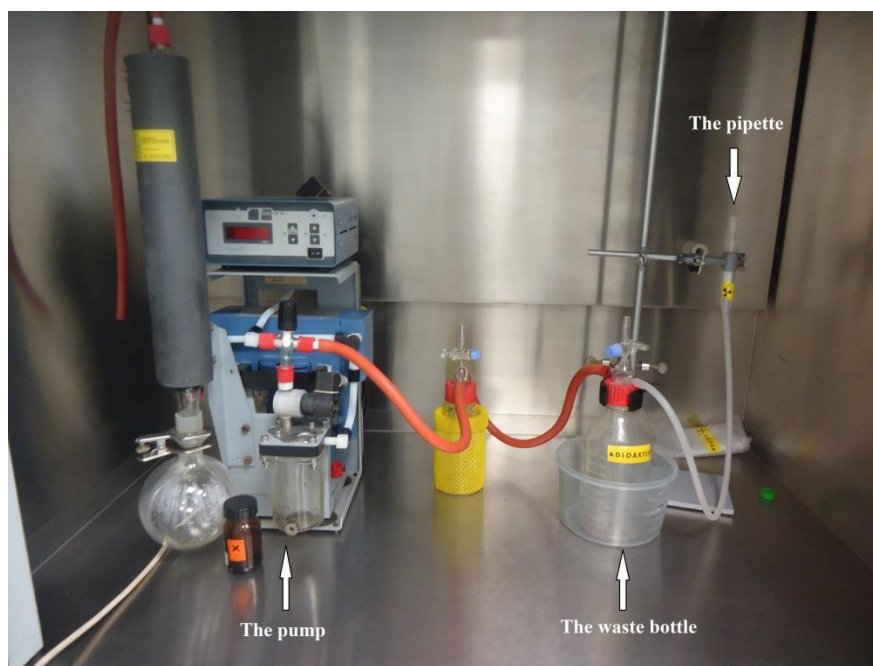
Figure 8: Apparatus used in the uptake assay

As mentioned before we tried to develop an assay with 1321N1 astrocytoma cells and HEK293 cells, because in those cell lines it was discovered that hENT1 and hENT2 transporters are expressed at high levels. However, experiments showed that HEK293 cells did not attach strongly enough on the bottom of the wells in the microplate. We observed that many cells were washed away during the experiment. This means that we did not have

equal conditions in each well and the results could not be trusted. Therefore, we decided to proceed the assay development only with 1321N1 astrocytoma cells. For the assay it was also highly important that cells are in good shape and confluent in each well. The different cells' concentration and incubation time were tested. It was observed that the best conditions are reached when seeding 6.5×10^5 cells/ well and incubating them for 2 day at 37 °C with humidified atmosphere of 5 % CO_2 and 95 % air.

After the cell line was chosen and equipment was prepared, we proceeded with investigating [^{3}H] adenine (30 nM) uptake by monitoring the time period of [^{3}H] adenine transport into 1321N1 astrocytoma cells (see time-depended assay protocol in chapter 3.2.8.1). Two independent experiments were performed in triplicates. As shown in Figure 9, adenine is transported in a time-dependent manner. In the first minutes the transport of adenine is very rapid; however a plateau is reached after 40 minutes. During the assay it is desired to have rapid transport of a ligand and a plateau should not be reached. Therefore, the incubation time with [^{3}H] adenine was set for 2 minutes.
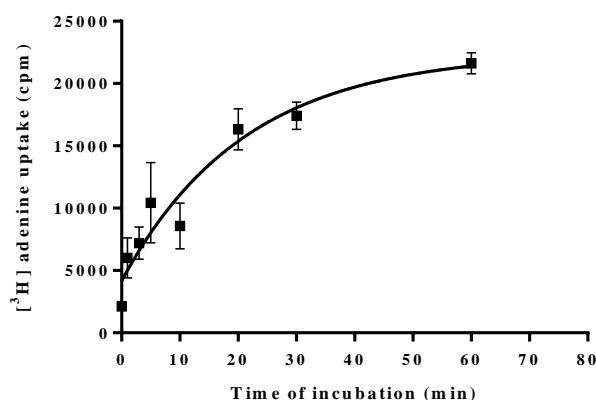
Figure 9: The time-depended [^{3}H] adenine transport in 1321N1 astrocytoma cells
[^{3}H] adenine (30 nM) was transported into 1321N1 astrocytoma cells for period of 1, 3, 5, 10, 20, 30, and 60 minutes. Data are presented as amount of radioactivity (cpm) depending to time of incubation (mean ± SEM; n=2).

At last the half maximal inhibitory concentration (IC_{50}) of a known inhibitor was determined with the inhibitor's concentration dependent assay (see protocol in chapter 3.2.8.1). As inhibitor we chose dipyridamole, which inhibits both hENT1 and hENT2, furthermore it is classified as a potent inhibitor. The assay was performed in triplicates of inhibitor concentration 0.005, 0.05, 0.5, 5, 25 and 50 µM (Figure 10) and sixfold positive control. [^{3}H] adenine (30 nM) was used as radioligand as in time-depended assay.
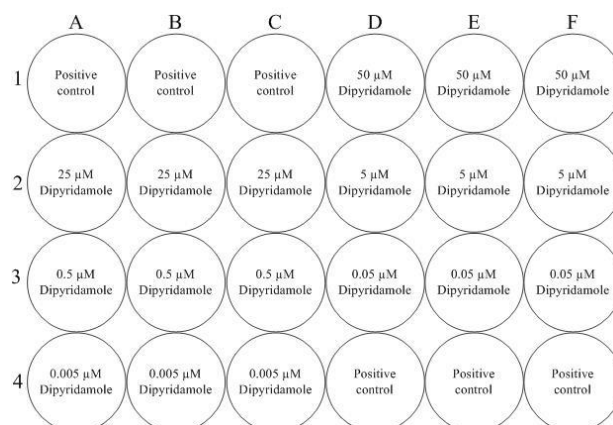
Figure 10: Example of 24-well plate layout

The example scheme shows the layout of 24-well plate when using the different concentration of dipyridamole (inhibitor). In all wells [^{3}H] adenine (30 nM) is used as radioligand for uptake period of 2 minutes. Assay is planned in triples for each inhibitor concentration and sixfold for positive control.

As expected the curve percent [^{3}H] adenine uptake as a function of the concentration of dipyridamole formed a typical inhibition curve, showing the drop of adenine transport at higher concentration of inhibitor. Moreover, percent of transporter's inhibition is changed rapidly at certain concentration of inhibitor. Dipyridamole inhibited adenine transport with IC$_{50}$ value of 0.496 nM (SEM $=\pm$ 0.082 nM). The IC$_{50}$ value was determined from the curve, which was drawn after 6 individual experiments performed in triplicates (Figure 11).
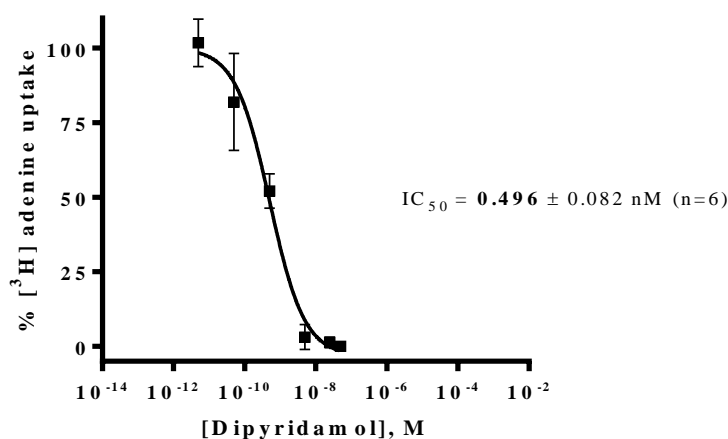
Figure 11: Concentration-dependent inhibition of adenine transport.

Graded concentrations of known inhibitor (dipyridamole) were incubated with 1321N1 astrocytoma cells for 30 minutes before adding of [^{3}H] adenine for an uptake period of 2 minutes. Data are presented as percent inhibition relative to the control (mean ± SEM; n=6).

To sum up, an uptake assay for characterisation of the hENT1 and the hENT2 was established on 1321N1 astrocytoma cells, because they attached strongly on the bottom of

the wells in the microtiter plate and they naturally express respective transporters. The inhibitor dipyridamole was tested in the newly established uptake assay and an IC_{50} value of 0.496 ± 0.082 nM was determined.

The pharmacological characterisation of the native transporters expressed in 1321N1 astrocytoma cell line could be followed by an assay examining the percent of adenine transport depending on adenine concentration. The Michaelis constant (K_m) and the maximum rate (v_{max}) for adenine could be calculated from the obtained data.

Furthermore, we could also characterise the transport of other nucleosides and nucleobases. We would first continue with uridine (pyrimidine nucleoside) and adenosine (purine nucleoside) uptake assay in order to cover different groups. Those assays could be established in a same way as adenine assay using same equipment and cell line.

4.1.2 GENERATION OF CELL LINES STABLY EXPRESSING hENT1 AND hENT2 TRANSPORTERS

The generation of a cell line stably expressing hENT1 and hENT2 was performed in 3 steps: 1) isolating full-length hENT1 and hENT2 cDNA, 2) construction of new plasmids, 3) generation of new cell lines stably expressing the respective transporter (Figure 7).

In the first step, the hENT1 and hENT2 mRNA were isolated from 1321N1 astrocytoma cells and HEK293 cells cultured in 6-well plates. The mRNA was then briefly treated with DNAse and then reverse transcribed into cDNA sequence using random hexamers with Superscript II RNase Reverse Transcriptase. The second DNA strand was built with designed ENT primers (Chapter 3.1.9) with PCR. In multiple cycles of PCR the amount of DNA was multiplied. The PCR product was cloned into the pJet vector. A chemocompetent bacterium was transformed and new recombinant plasmids were isolated and subjected to DNA sequencing.

The hENT1 and hENT2 specific primers, which were designed and ordered prior the experiment, were used in PCR. Unfortunately, we have faced several problems in designing primers. Therefore, the primers were designed not to anneal at the beginning of transporter's sequence, but more in the middle.

As it is evident from sequencing result (Chapter 7.1) we have managed to isolate only part of the hENT1 and hENT2 transporters. The cloned part of the hENT1 transporter is 403

amino acids long, which means that 53 amino acid sequence is missing at the beginning. Unfortunately, 6 mutations were found in cloned sequence of hENT1. On the other hand, there were no mutation in cloned sequence of the hENT2 transporter; however we only managed to clone 298 amino acids long sequence (Figure 12). Taken together, even though we planned our primers to anneal within the cDNA sequence, more residues are missing at 5'-end than expected (11 residues were expected to be missed in hENT1 and 22 residues in hENT2). Apparently, the residues in the middle enabled our primers to anneal somewhere else due to high homology.
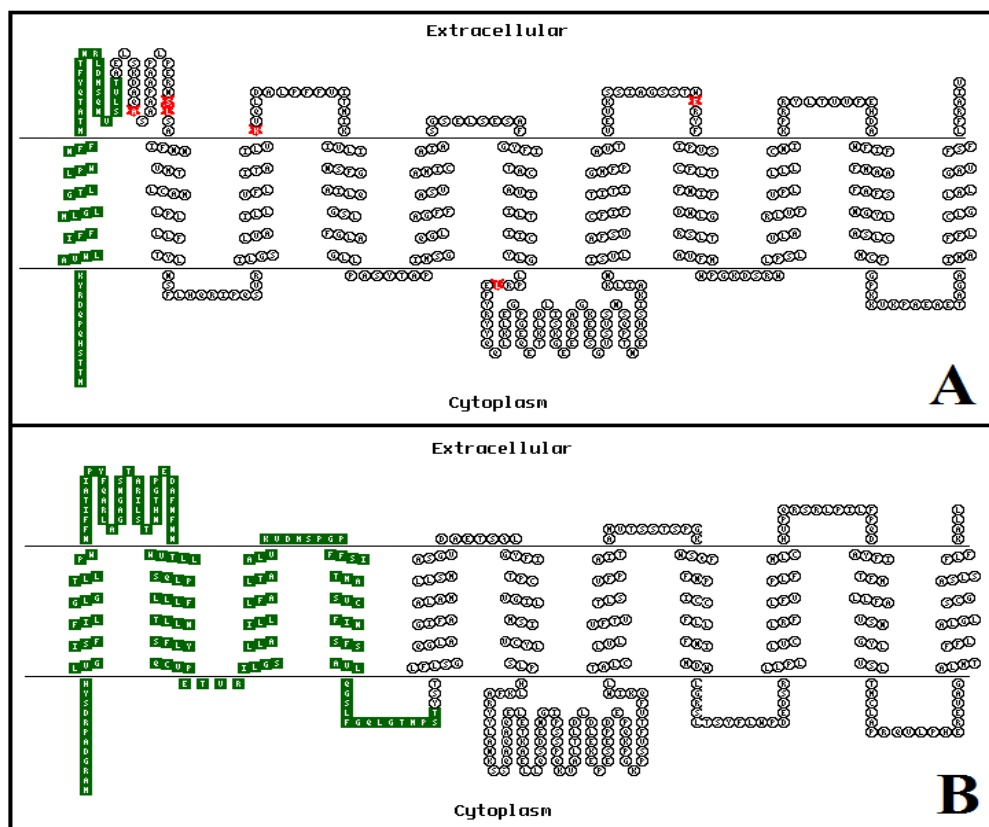
Figure 12: hENT1 and hENT2 predicted topology model
The snake-plot model was drawn with TOPO2 for hENT1 (A) and hENT2 (B). TMHMM was used for prediction of transmembrane helices. The residues coloured in green show amino acids, which are missing in our cloned transporters cDNA, and the residues coloured in red show the mismatches in our cloned hENT1 protein.

As shown in Figure 12, essential parts of hENT1 and hENT2 are missing in our sequence. Therefore, we need to add the missing parts of the transporters and remove mismatched amino acids in hENT1 prior continuing with construction of new plasmids. Our original idea was to add missing residues with primers, which will anneal at 5'-end and have an overhang of 15-24 nucleotides. With the overhang missing 5-8 amino acids could be added. However

being in the present situation, we thought about designing new primers and proceed with cloning of the second part of the sequence. Two parts will be in the end ligated together. Unfortunately, due to the time limitation we did not succeed with generation of full-length hENT1 and hENT2 cDNA. Therefore, we could not continue with plasmid construction and generation of CHO cell line stably expressing hENT1 and hENT2. However, with cloning it was confirmed that both hENT1 and hENT2 transporters are expressed in 1321N1 astrocytoma cells and HEK293 cells.

4.2   mMrgA PROJECT

In the mMrgA project the aims were to clone mMrgA1, mMrgA2b and mMrgA6 receptor genes into new vectors and conduct its expression in CHO cell lines. After establishment of a CHO cell line stably expressing the respective receptor, the screening of possible ligands shall be performed using the cAMP binding assay.

The Figure 13 presents the overview of the research process.
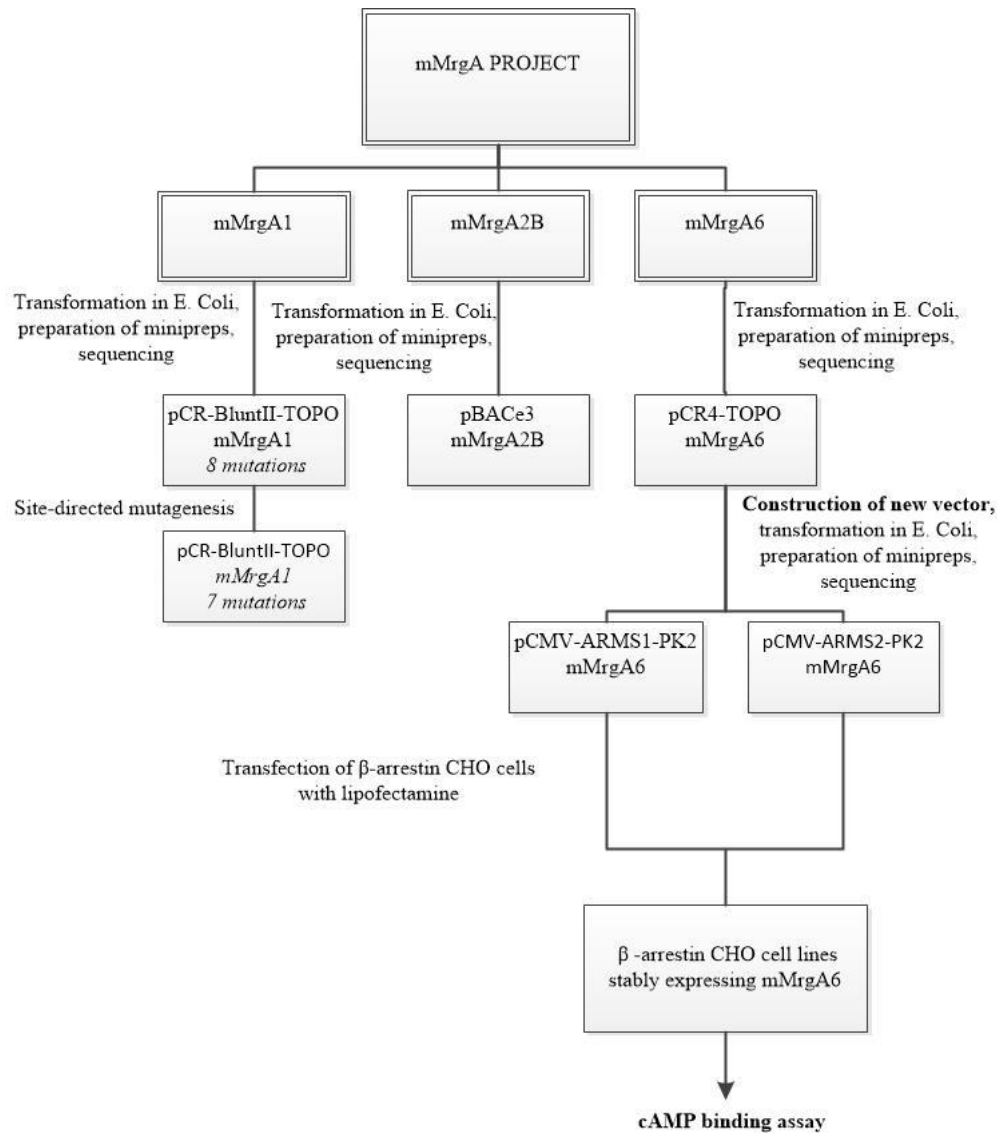
Figure 13: Research plan of mMrgA project

4.2.1 CLONING OF mMrgA1, mMrgA2B AND mMrgA6 GENES

mMrgA genes were bought in vectors, which could be expressed in bacterial cells (Table XVI). Our first step was to transform *E. coli* with all the genes and to isolate DNA by preparing mini preparations. Samples were then sent to sequencing. Sequencing results were aligned with reference sequences found in PubMed using Clustal W2 program in order to check if sequence meet our requirements.

Table XVI: List of original vectors containing cDNA of mMrgA receptors

Vector	Receptor	Size of vector	Resistance
pCR-Blunt II-TOPO	mMrgA1	3519 bp	kanamycin
pBACe3	mMrgA2b	11612 bp	chloramphenicol
pCR4-TOPO	mMrgA6	3956 bp	kanamycin

The **mMrgA1** cDNA was successfully cloned, however alignment between our cloned sequence and reference sequenced showed 8 mutations (Chapter 7.1). Before continuing with expression into new vector we decided to remove mutation with site-directed mutagenesis, which proved to be a very difficult process in the case of this gene (Chapter 4.2.2).

Unfortunately, we have faced several problems in cloning **mMrgA2b** gene. Even though different methods and kits were tried out, we could not isolate the cDNA. Contradictory, when using a thin layer chromatography to check successful preparation of mini-scale or midi-scale preparations, the band was seen on the agarose gel; however we have not received any results from GATC sequencing company claiming that samples did not contain any DNA. At present we cannot explain what happened to the DNA.

Anyway, in the end we decided to send our sample to sequence as linear DNA. Sequencing linear cDNA has few disadvantages in comparison to sequencing circular DNA. Firstly, we needed to be provided the primers, which was not a problem in our case (Chapter 3.1.9). Moreover, there are more background peaks, which means that results are less reliable. As shown in Figure 14 the background was a problem in our reaction and might be also the reason of numerous mutation found in alignment with the reference sequence. However, we manage to confirm the identity of our mMrgA2b gene with BLAST® tool.

In future, the PCR product could be subcloned into pcDNA3.1(+) vector. The DNA mini-scale preparations could be prepared and subjected to sequencing reaction. With this procedure, we could find out if the problem with sequencing is eliminated. Furthermore, also

more reliable sequence information should be obtained. Therefore, we can make alignment with reference sequence again and hopefully less mutation will be found.
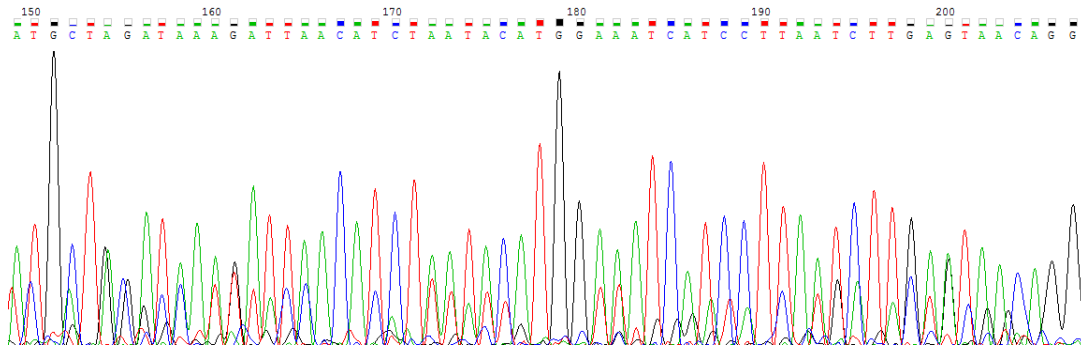
Figure 14: Part of sequencing results for mMrgA2b

The Figure shows part of result for sequencing reaction of mMrgA2b. The residues on position 149-207 are shown (see first row). Each peak should represent one residue, however if two peaks are annealed the amino acid, which is represented by higher peak, is included in the sequence. The higher the background peak is less reliable the results are. The certainty of residues is shown in the squares (second row), whereas the full square represents more certainty than empty square.

The **mMrgA6** cDNA was successfully cloned as well. In alignment with reference sequence (Chapter 7.1) no mutations were found, so we continued with subcloning in Prolink vectors pCMV-ARMS1-PK2 and pCMV-ARMS2-PK2. This step was necessary, because vector pCR4-TOPO, in which gene was bought, can be only expressed in bacterial cells and our aim was to establish stable mammalian cell lines.

In conclusion, the objective to clone mMrgA1, mMrgA2b and mMRgA6 was partly reached: the full-length of mMrgA6, mMrgA2b and mMrgA1 gene was obtained, the latter contains 8 mismatched amino acids, so continuation with site-directed mutagenesis is needed in order to get native gene. The mMrgA2b DNA should be sent to sequencing again, in order to receive more reliable results to draw conclusions.

4.2.2 SITE-DIRECTED MUTAGENESIS

Since 8 point mutations were found in mMrgA1 gene (Figure 15), we proceeded with site-directed mutagenesis. Firstly, mutagenesis oligonucleotide primers were designed containing the corresponding mutation and ordered at Invitrogen[TM] by *life* technologies[TM] company (Chapter 3.1.9). Site-directed mutagenesis was performed as described under methods (Chapter 3.2.6.3) followed by digestion with the restriction enzyme D*pnI*.

One cycle is needed for removal of one mutation; therefore, eight successive cycles were planned. After each cycle gene was transformed into competent *E. coli* cells in order to prepare isolation of single colonies and send samples for sequencing to check if mutation was successfully removed.

The sequence of the gene has such a characteristic that good primers for site-directed mutagenesis were very difficult to design. Therefore, we have experienced several problems in the process and in the end only mutation on position 102 (marked in green in Figure 15) was successfully removed.
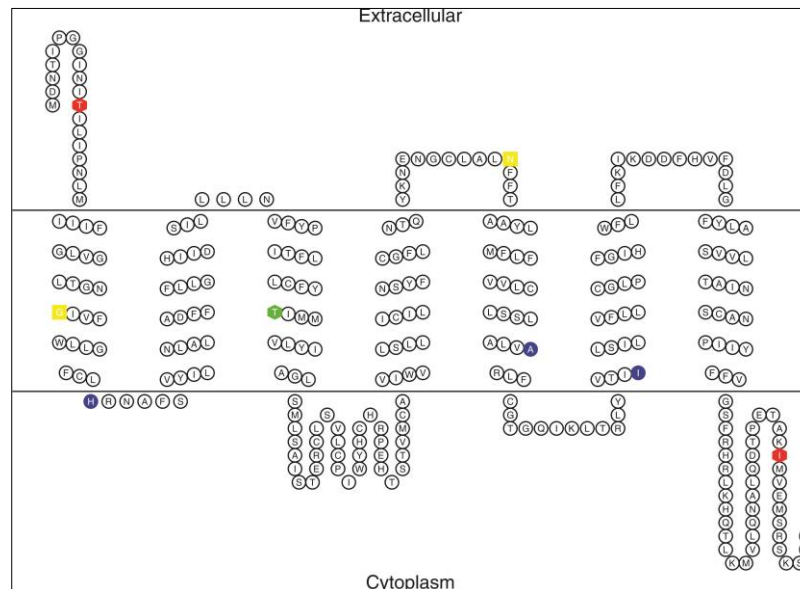
Figure 15: mMrgA1 predicted topology model

The snake-plot model was drawn with TOPO2. TMHMM was used for prediction of transmembrane helices. The residues coloured show mutations found in our cloned gene as follows: blue indicates strong similarity; yellow indicates weak similarity and red indicates no similarity between mismatched residues. Green colour indicates successfully removed mutation.

The site-directed mutagenesis should be continued with the adjustments applied. We have always used the same PCR protocol (Chapter 3.2.6.3), even though melting temperatures (T_m) of primers were different (Chapter 3.1.9). The literature suggests that the primers anneal the best at the temperature T_m-2 °C, so we should adapt the PCR protocol to each primer.

4.2.3 CONSTRUCTION OF NEW PLASMIDS

We wanted to subclone the mMrgA6 gene into Prolink vectors (pCMV-ARMS1-PK2 and pCMV-ARMS2-PK2). Restriction cleavage with BglI and NheI-HF restriction enzymes of pCR-Blunt II-TOPO mMrgA6 and Prolink vector was the first step followed by dephosphorylation of cut plasmid and ligation of the protein and the Prolink vector. Transformation, isolation and sequencing of the newly constructed plasmid were performed. After alignment of the sequencing results with the reference sequence it was proved that two new plasmids, ARMS1 mMrgA6 and ARMS2 mMrgA6, were successfully generated without any mutation. Therefore, we continued with the preparation of midi preparations to

multiply the amount of DNA in the sample. The higher concentration of DNA in the sample is needed for successful transfection of the mammalian cells.
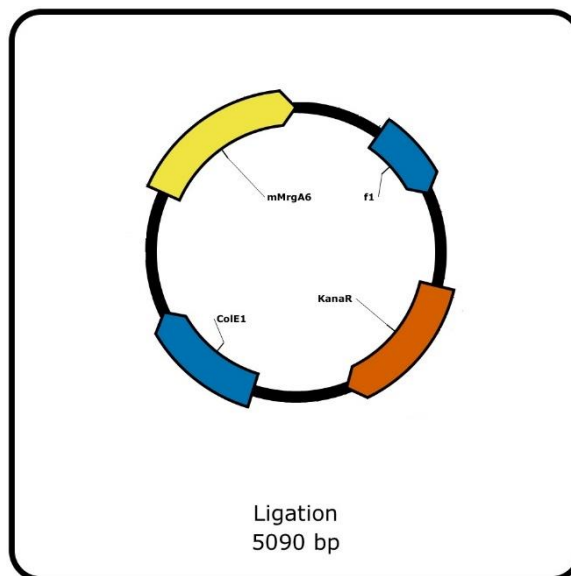
Figure 16: Schematic presentation of newly generated vector
The ligation of the pCMV-ARMS1-PK2 and the mMrA6 generated new vector.

4.2.4 TRANSFECTION OF MAMMALIAN CELLS

The stably transfected cell lines were established in β-arrestin CHO cells by lipofection with Lipofectamine 2000 as described under methods (Chapter 3.2.7.4). The selection was performed with antibiotic G418 for 12 days. The end point was determined when control cells, which were not stably expressing mMrgA6, died. After selection process, we had two stable β-arrestin CHO cell lines with mMrgA6 receptor, which were used for cAMP binding assay.

In addition, we have also generated CHO-K1 cells recombinantly expressing mMrgA6, however those lines were not stable, but only generated to perform preliminary cAMP assay (data not shown). The transfection steps were the same, however the cells were not threaded with G418 and were immediately seeded for the assay.

After cells transfection with the cDNA of our interest, it is always important to check if the process was successful before continuation. Both methods described above for production of cells expressing respective protein have high level of reliability that protein was successfully expressed and this could be checked in several ways. In our case, we cannot confirm that cells were successfully transfected in CHO-K1 cells. On the other hand, β-arrestin CHO cells went through selection with G418, which shows that cells are expressing mMrgA6, therefore we continued with assay preparation.

In addition, it would have been beneficial to evaluate expression level of protein as well. The suggested method would be the Western Blot.

4.2.5 MEASUREMENT OF cAMP LEVEL

To date the ligand for mMrgA6 is not known yet. The idea is that cAMP assay will be used for screening of potential agonists. In our studies, we have started the screening with adenine, which is suggested to be a ligand of mMrgA subfamily (19).

It is assumed that mMrgA6 signals through G_i pathways, therefore the respective protocol for cAMP binding assay was used (Chapter 3.2.8.2).

The adenine molecule was able to inhibit adenyl cyclase activity and to decrease cAMP levels in β-arrestin CHO cells stably expressing mMrgA6. The calculated half maximal effective concentration (EC_{50}) was 977 nM, which showed quite good potency of adenine at mMrgA6 receptor. As shown in Figure 17 and Figure 18 cAMP level were decreased, however only for 7.75 pmol (31.0 %).
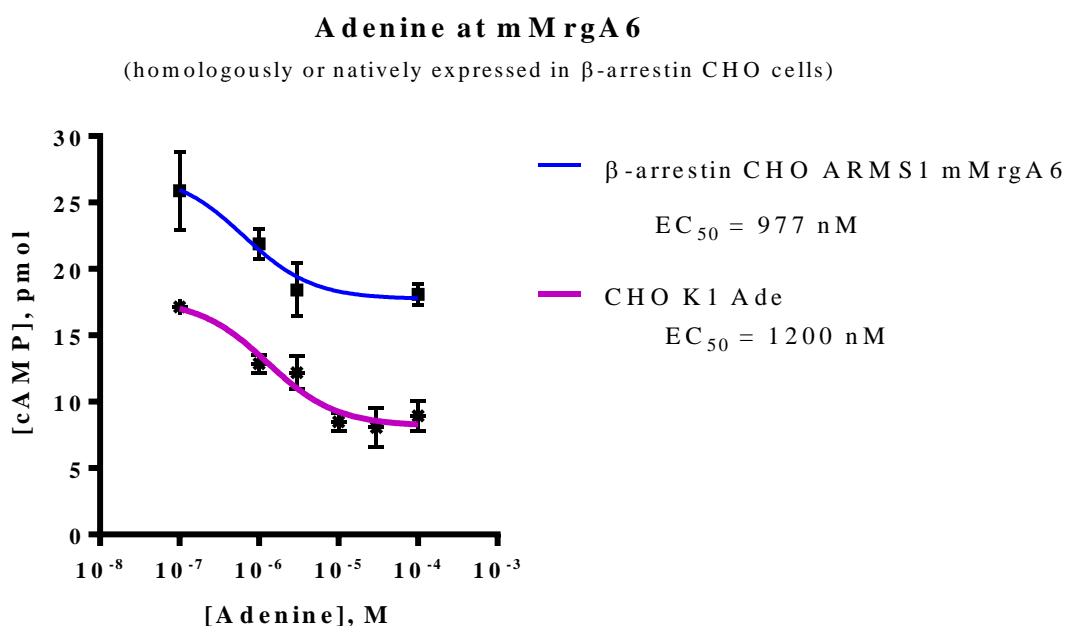
Figure 17: Concentration-response curve for cAMP accumulation assays.
Concentration-response curves show mean cAMP accumulation in CHO cells recombinantly expressing mMrgA6 (maximally accumulated cAMP amounts induced by 10 μM forskolin) after receptor activation by adenine. Data represent means ± SEM of one independent experiment.
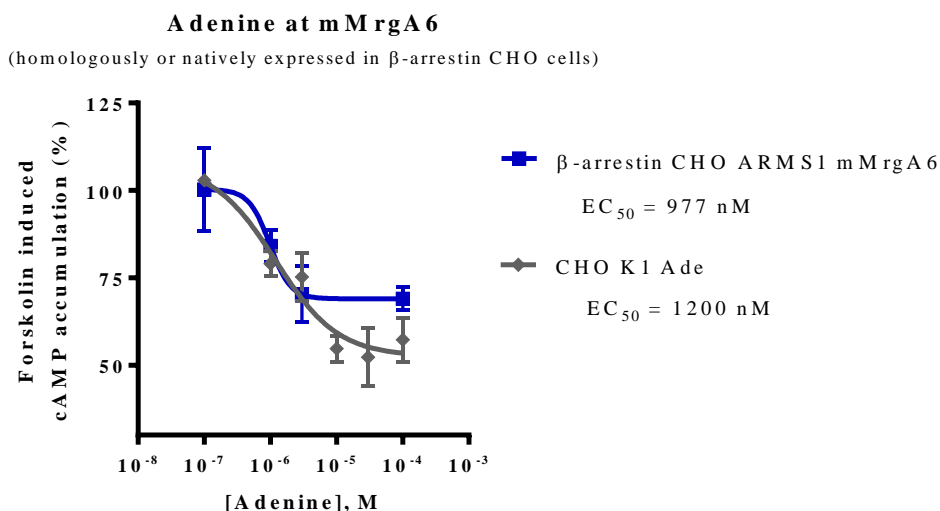
Figure 18: Concentration-response curve for cAMP accumulation assays

Concentration-response curves show mean cAMP accumulation in CHO cells recombinantly expressing mMrgA6 (in percent maximally accumulated cAMP amounts (~24 pmol) induced by 10 μM forskolin) after receptor activation by adenine. Data represent means $\pm$ SEM of one independent experiment

Unfortunately, the use of negative or positive control in this assay was impossible. However, we used adenine receptor expressed in CHO-K1 cell line prepared by Dominik Thimm, PhD student in the prof. Müller's research group, as control of the assay protocol. The EC_{50} was measured 1200 nM, which is comparable to his results, suggesting the assay was handled appropriately (Figure 17).

When expressed in β-arrestin CHO cells, these novel fusion proteins did function as receptors mediating a decrease in cellular cAMP levels, however the decrease was not significantly relevant. There is also a need to perform more assays to confirm the conclusion and to determine EC_{50} more precisely.

5   CONCLUSION

Nucleobase transporters and receptors are the best entry pathways for most nucleobase and nucleoside – based medicines, currently available on the market. However, the challenge remains since complete pharmacological profiles and molecular determinants of transportability are not yet fully known (2).

This master thesis discusses a background, the research methods and results of cloning, expression and pharmacological characterisation of human ENT transporters and mouse MrgA receptors.

The mouse Mas related G-protein coupled receptor member A1, member A2b and member A6 were successfully isolated and cloned, however mMrgA1 sequence still contains seven point mutations. In addition, also parts of human equilibrative nucleoside transporter 1 and human equilibrative nucleoside transporter 2 were successfully cloned. Our objective was partly reached, so the work should be continued to obtain mMrgA1 and mMrgA2b cDNA without point mutation and hENT1 and hENT2 full-length cDNA.

Furthermore, mMrgA6 was successfully ligated with Prolink vectors: pCMV-ARMS1-PK2 and pCMV-ARMS2-PK2 and stably expressed in β-arrestin CHO cell line. The cAMP assay through G_i pathways and β-arrestin assay were established for screening of potential agonists. Adenine was tested and it decreased the cAMP level, however not significantly. Therefore, we cannot conclude if adenine acts as full or partial agonist without further assays. Moreover, pharmacological assay was also established at 1321N1 astrocytoma cells for characterisation on hENT1 and hENT2. The assay parameters, namely time of incubation and half maximal inhibitory concentration (IC_{50}), were determined. The assay is now prepared for screening potential inhibitors of hENT1 and hENT2. In the future, the assay could be established on β-arrestin CHO cell line stably expressing respective transporter. With this assay, we would be able to distinguish inhibitors' effect on different subtypes of hENT.

As it is evident from the master thesis, handling with nucleobase transporters and receptors is not easy and we have faced many challenges in the development of *in vitro* pharmacological assays. The respective proteins are long polynucleotides and they have many homologous areas, therefore adapted molecular biology tools should be applied.

We conclude that developed uptake assay on 1321N1 astrocytoma cells is reliable for screening the library of proposed inhibitors of hENT. The possibility to calculate IC_{50} will enable to summarise screening results as SAR formula. Furthermore, cAMP binding assay and β-arrestin assay could be used for screening agonist and antagonist of mMrgA6. Nevertheless, we recommend further investigations and improvements on both proposed assays.

To conclude, there are many possibilities for future researches in area of nucleobase transporters and receptors in order to fully understand their role in physiological and (patho) physiological processes, which help us design new medicines.

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Website used in development of master thesis:

- TOPO2 Transmembrane Protein Display Page

   http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py (27.11.2014)

- Clustal W2

   http://www.ebi.ac.uk/Tools/msa/clustalw2 (27.11.2014)

- TMHMM Server v. 2.0
  http://www.cbs.dtu.dk/services/TMHMM (27.11.2014)
- BLAST: Basic Local Alignment Search Tool
  http://blast.ncbi.nlm.nih.gov/Blast.cgi (27.11.2014)

7   APPENDICES

7.1   SEQUENCE ALIGMENTS

hENT1 (reference: http://www.ncbi.nlm.nih.gov/protein/NP_001071645.1)

```
ENT1_NCBI      MTTSHQPQDRYKAVWLIFFMLGLGTLLPWNFFMTATQYFTNRLDMSQNVSLVTAELSKDA 60
ENT1_clonned   ----------SKRCWVYLFQAGFGNRVPRGIFHGGPSSFPKPPGHVPEWSLVIAELSKDA 50
                         *  *: :*  *:*. :* .:*  ... *.:  .   : *** *******

ENT1_NCBI      QASAAPAAPLPERNSLSAIFNNVMTLCAMLPLLLFTYLNSFLHQRIPQSVRILGSLVAIL 120
ENT1_clonned   QGSAAPAAPLPERNFFSAIFNNVMTLCAMLPLLLFTYLNSFLHQRIPQSVRILGSLVAIL 110
               *.************ :********************************************

ENT1_NCBI      LVFLITAILVKVQLDALPFFVITMIKIVLINSFGAILQGSLFGLAGLLPASYTAPIMSGQ 180
ENT1_clonned   LVFLITAILVRVQLDALPFFVITMIKIVLINSFGAILQGSLFGLAGLLPASYTAPIMSGQ 170
               **********:*************************************************

ENT1_NCBI      GLAGFFASVAMICAIASGSELSESAFGYFITACAVIILTIICYLGLPRLEFYRYYQQLKL 240
ENT1_clonned   GLAGFFASVAMICAIASGSELSESAFGYFITACAVIILTIICYLGLPRPEFYRYYQQLKL 230
               *********************************************** ***********

ENT1_NCBI      EGPGEQETKLDLISKGEEPRAGKEESGVSVSNSQPTNESHSIKAILKNISVLAFSVCFIF 300
ENT1_clonned   EGPGEQETKLDLISKGEEPRAGKEESGVSVSNSQPTNESHSIKAILKNISVLAFSVCFIF 290
               ************************************************************

ENT1_NCBI      TITIGMFPAVTVEVKSSIAGSSTWERYFIPVSCFLTFNIFDWLGRSLTAVFMWPGKDSRW 360
ENT1_clonned   TITIGMFPAVTVEVKSSIAGSSTWGRYFIPVSCFLTFNIFDWLGRSLTAVFMWPGKDSRW 350
               ************************ ***********************************

ENT1_NCBI      LPSLVLARLVFVPLLLLCNIKPRRYLTVVFEHDAWFIFFMAAFAFSNGYLASLCMCFGPK 420
ENT1_clonned   LPSLVLARLVFVPLLLLCNIKPRRYLTVVFEHDAWFIFFMAAFAFSNGYLASLCMCFGPK 410
               ************************************************************

ENT1_NCBI      KVKPAEAETAGAIMAFFLCLGLALGAVFSFLFRAIV- 456
ENT1_clonned   KVKPAEAETAGAIMAFFLCLGLALGAVFSFLFRAIV- 446
               ***********************************
```

hENT2 (reference: http://www.ncbi.nlm.nih.gov/protein/NP_001287797.1)

```
ENT2_NCBI      MARGDAPRDSYHLVGISFFILGLGTLLPWNFFITAIPYFQARLAGAGNSTARILSTNHTG 60
ENT2_clonned   -----------------------SRRRCTFWAACWPYCCSLP-------QQRWSRWT-A 28
                                      :    .*: :. ** :        : *   .

ENT2_NCBI      PEDAFNFNNWVTLLSQLPLLLFTLLNSFLYQCVPETVRILGSLLAILLLFALTAALVKVD 120
ENT2_clonned   PDPSF------------------------------------------------------ 33
               *: :*

ENT2_NCBI      MSPGPFFSITMASVCFINSFSAVLQGSLFGQLGTMPSTYSTLFLSGQGLAGIFAALAMLL 180
ENT2_clonned   --PSP-WPPSASSTPSVQSYRAASS----GSWAPCPPPYSTLFLSGQGLAGIFAALAMLL 86
                 *.* :. : :*.  ::*: *. .    *. .. *..*******************

ENT2_NCBI      SMASGVDAETSALGYFITPCVGILMSIVCYLSLPHLKFARYYLANKSSQAQAQELETKAE 240
ENT2_clonned   SMASGVDAETSALGYFITPCVGILMSIVCYLSLPHLKFARYYLANKSSQAQAQELETKAE 146
               ************************************************************

ENT2_NCBI      LLQSDENGIPSSPQKVALTLDLDLEKEPESEPDEPQKPGKPSVFTVFQKIWLTALCLVLV 300
ENT2_clonned   LLQSDENGIPSSPQKVALTLDLDLEKEPESEPDEPQKPGKPSVFTVFQKIWLTALCLVLV 206
               ************************************************************

ENT2_NCBI      FTVTLSVFPAITAMVTSSTSPGKWSQFFNPICCFLLFNIMDWLGRSLTSYFLWPDEDSRL 360
ENT2_clonned   FTVTLSVFPAITAMVTSSTSPGKWSQFFNPICCFLLFNIMDWLGRSLTSYFLWPDEDSRL 266
               ************************************************************
```

```
ENT2_NCBI        LPLLVCLRFLFVPLFMLCHVPQRSRLPILFPQDAYFITFMLLFAVSNGYLVSLTMCLAPR 420
ENT2_clonned     LPLLVCLRFLFVPLFMLCHVPQRSRLPILFPQDAYFITFMLLFAVSNGYLVSLTMCLAPR 326
                 ***********************************************************


ENT2_NCBI        QVLPHEREVAGALMTFFLALGLSCGASLSFLFKALL- 456
ENT2_clonned     QVLPHEREVAGALMTFFLALGLSCGASLSFLFKALL- 362
                 ************************************
```

mMrgA1 (reference: http://www.ncbi.nlm.nih.gov/protein/AAI26874.1)

```
mMrgA1_NCBI      MDNTIPGGINIRILIPNLMIIIFGLVGLTGNAIVFWLLGFCLRRNAFSVYILNLALADFF 60
mMrgA1_cloned    MDNTIPGGINIRILIPNLMIIIFGLVGLTGNAIVFWLLGFCLRRNAFSVYILNLALADFF 60
                 ************************************************************

mMrgA1_NCBI      FLLGHIIDSILLLLNVFYPITFLLCFYIIMMVLYIAGLSMLSAISTERCLSVLCPIWYHC 120
mMrgA1_cloned    FLLGHIIDSILLLLNVFYPITFLLCFYIIMMVLYIAGLSMLSAISTERCLSVLCPIWYHC 120
                 ************************************************************

mMrgA1_NCBI      HRPEHTSTVMCAVIWVLSLLICILNSYFCGFLNTQYKNENGCLALSFFTAAYLMFLFVVL 180
mMrgA1_cloned    HRPEHTSTVMCAVIWVLSLLICILNSYFCGFLNTQYKNENGCLALSFFTAAYLMFLFVVL 180
                 ************************************************************

mMrgA1_NCBI      CLSSLALVSRLFCGTGQIKLTRLYVTIMLSILVFLLCGLPFGIHWFLLFKIKDDFHVFDL 240
mMrgA1_cloned    CLSSLALVSRLFCGTGQIKLTRLYVTIMLSILVFLLCGLPFGIHWFLLFKIKDDFHVFDL 240
                 ************************************************************

mMrgA1_NCBI      GFYLASVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQNALQDTPETAKNMVEMSRS 300
mMrgA1_cloned    GFYLASVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQNALQDTPETAKNMVEDVKK 300
                 ****************************************************     :.

mMrgA1_NCBI      KSEP- 304
mMrgA1_cloned    QIKAM 305
                 : :.
```

mMrgA1 (reference: http://www.ncbi.nlm.nih.gov/nuccore/AY042191.1)- *8 mutations*

```
mMrgA1_NCBI      PGGINITILIPNLMIIIFGLVGLTGNGIVFWLLGFCLHRNAFSVYILNLALADFFFLLGH 60
mMrgA1_cloned    PGGINIRILIPNLMIIIFGLVGLTGNAIVFWLLGFCLRRNAFSVYILNLALADFFFLLGH 60
                 ******.*******************.***********:*********************

mMrgA1_NCBI      IIDSILLLLNVFYPITFLLCFYTIMMVLYIAGLSMLSAISTERCLSVLCPIWYHCHRPEH 120
mMrgA1_cloned    IIDSILLLLNVFYPITFLLCFYIIMMVLYIAGLSMLSAISTERCLSVLCPIWYHCHRPEH 120
                 ********************** *************************************

mMrgA1_NCBI      TSTVMCAVIWVLSLLICILNSYFCGFLNTQYKNENGCLALNFFTAAYLMFLFVVLCLSSL 180
mMrgA1_cloned    TSTVMCAVIWVLSLLICILNSYFCGFLNTQYKNENGCLALSFFTAAYLMFLFVVLCLSSL 180
                 ****************************************.*******************

mMrgA1_NCBI      ALVARLFCGTGQIKLTRLYVTIILSILVFLLCGLPFGIHWFLLFKIKDDFHVFDLGFYLA 240
mMrgA1_cloned    ALVSRLFCGTGQIKLTRLYVTIMLSILVFLLCGLPFGIHWFLLFKIKDDFHVFDLGFYLA 240
                 ***:******************:************************************

mMrgA1_NCBI      SVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQNALQDTPETAKIMVEMSRSKSEP- 299
mMrgA1_cloned    SVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQNALQDTPETAKNMVEDVKKQIKAM 300
                 *********************************************** ***  :.: :.
```

mMrgA2b (reference: http://www.ncbi.nlm.nih.gov/protein/Q91WW4.1) - *35 mutations*

```
mMrgA2_NCBI      MDETLPGSINIRILIPKLMIIIFGLVGLMGNAIVFWLLGFHLRRNAFSVYILNLALADFL 60
mMrgA2b_cloned   MDETLPGSINIESLIPKLMSIIFGLVGLMGNAIVFWLLGFHLRRNAFSVYILNLALADFL 60
                 ***********.  ****** *************************************
```

```
mMrgA2_NCBI        FLLSSIIASTLFLLKVSYLSIIFHLCFNTIMMVVYITGISMLSAISTECCLSVLCPTWYR 120
mMrgA2b_cloned     FLLSSIIPSTLFLLEVSYLSIIFHLCFNTIMMVVYVTGISMLELIKTECCLSVLSPTWYP 120
                   *******.******:***********************:******. *.********.****

mMrgA2_NCBI        CHRPVHTSTVMCAVIWVLSLLICILNSYFCAVLHTRYDNDNECLATNIFTASYMIFLLVV 180
mMrgA2b_cloned     CHRPVHTSTVMCDVIWVLSLLICILNSYFCAVLHTKYHNDNDCLATNNFTASDMIFLLVI 180
                   ************ ***********************:*.***:***** **** ******:

mMrgA2_NCBI        LCLSSLALLARLFCGAGQMKLTRFHVTILLTLLVFLLCGLPFVIYCILLFKIKDDFHVLD 240
mMrgA2b_cloned     LCLSRLALLATLFCGAGHMKITRFHVTILLTLLIFLLCMMPFVHYCIQLLKINDDFHVLD 240
                   **** ***** ******:**:************:**** :*** *** *:**:*******

mMrgA2_NCBI        VNFYLALEVLTAINSCANPIIYFFVGSFRHQLKHQTLKMVLQSALQDTPETAENMVEMSS 300
mMrgA2b_cloned     VNLYLALQVLTAINSCANPIIYVFVGSFTRQLKHQTLKMVLQSALQDTPETAENMVEMSS 300
                   **:****:*************.***** :*************************** *****

mMrgA2_NCBI        NKAEP- 305
mMrgA2b_cloned     NKAESL 306
                   ****.
```

mMrgA6 (reference: http://www.ncbi.nlm.nih.gov/protein/AAI32205.1)

```
mMrgA6_NCBI        MHRSISIRILITNLMIVILGLVGLTGNAIVFWLLLFRLRRNAFSIYILNLALADFLFLLC 60
mMrgA6_cloned      MHRSISIRILITNLMIVILGLVGLTGNAIVFWLLLFRLRRNAFSIYILNLALADFLFLLC 60
                   ************************************************************

mMrgA6_NCBI        HIIASTEHILTFSSPNSIFINCLYTFRVLLYIAGLSMLSAISIERCLSVMCPIWYRCHSP 120
mMrgA6_cloned      HIIASTEHILTFSSPNSIFINCLYTFRVLLYIAGLSMLSAISIERCLSVMCPIWYRCHSP 120
                   ************************************************************

mMrgA6_NCBI        EHTSTVMCAMIWVLSLLLCILYRYFCGFLDTKYEDDYGCLAMNFLTTAYLMFLFVVLCVS 180
mMrgA6_cloned      EHTSTVMCAMIWVLSLLLCILYRYFCGFLDTKYEDDYGCLAMNFLTTAYLMFLFVVLCVS 180
                   ************************************************************

mMrgA6_NCBI        SLALLARLFCGAGRMKLTRLYVTITLTLLVFLLCGLPCGFYWFLLSKIKNVFTVFEFSLY 240
mMrgA6_cloned      SLALLARLFCGAGRMKLTRLYVTITLTLLVFLLCGLPCGFYWFLLSKIKNVFTVFEFSLY 240
                   ************************************************************

mMrgA6_NCBI        LASVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQSALQDTPETPENMVEMSMRNAAE 300
mMrgA6_cloned      LASVVLTAINSCANPIIYFFVGSFRHRLKHQTLKMVLQSALQDTPETPENMVEMSMRNAAE 300
                   ************************************************************

mMrgA6_NCBI        L- 301
mMrgA6_cloned      L- 301
                   *
```

7.2   AMINO ACIDS ABBREVIATIONS

Table XVII: List of 1- and 3-letters abbreviations of amino acids
References: http://en.wikipedia.org/wiki/Amino_acid

Amino Acid	3-Letters Abbreviation	1-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E

Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Valine	Val	V

7.3   GENETIC CODE

Table XVIII: Genetic code

The genetic code consists of 64 triplets of nucleotides (codon). With three exceptions, each codon encodes for one of 20 amino acids. The left-hand column gives the first nucleotide of the codon, the four middle columns give the second mucleotide, and the last column gives the third nucleotide. Reference: http://en.wikipedia.org/wiki/Genetic_code

Second Position

First Position [5' end]		U	C	A	G	Third Position [3' end]
	U	Phe	Ser	Tyr	Cys	**U**
		Phe	Ser	Tyr	Cys	**C**
		Leu	Ser	*Stop*	*Stop*	**A**
		Leu	Ser	*Stop*	Trp	**G**
	C	Leu	Pro	His	Arg	**U**
		Leu	Pro	His	Arg	**C**
		Leu	Pro	Gln	Arg	**A**
		Leu	Pro	Gln	Arg	**G**
	A	Ile	Thr	Asn	Ser	**U**
		Ile	Thr	Asn	Ser	**C**
		Ile	Thr	Lys	Arg	**A**
		Met or *Start*	Thr	Lys	Arg	**G**
	G	Val	Ala	Asp	Gly	**U**
		Val	Ala	Asp	Gly	**C**
		Val	Ala	Glu	Gly	**A**
		Val	Ala	Glu	Gly	**G**