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Identification and characterization of G protein-coupled nucleotide and nucleotide-like receptors P2Y13 and GPR17

(Diploma thesis)

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DECLARATION

I hereby declare that this diploma thesis was done by me under supervision of Dr. Aliaa Abdelrahman, Prof. Dr. Christa E. Müller and Prof. PharmDr. Martin Doležal, Ph.D. The literature used in the presented work is properly cited and listed in the resources section. This work was not used to obtain the same or other title.

Student's signature: ……...

ABSTRACT

Identification and characterization of G protein-coupled nucleotide and nucleotide-like receptors P2Y13 and GPR17

Purinergic receptors are divided into three subfamilies, P1 (adenosine), P2 (nucleotide) and P0 (adenine) receptors. P2 receptors comprise ligand-gated ion channels - shortly LGICs, receptors $P2X_{1\text{-}7}$, and class A, δ-branch GPCRs termed $P2Y_{1,2,3,6,12\text{-}14}$. The $P2Y_{13}$ receptor is physiologically activated by ADP and coupled to G_i whose activation results in an inhibition of adenylate cyclase. $P2Y_{13}$ receptor activation has been reported to be neuroprotective. $P2Y_{13}$ receptors also play a role in lipoprotein metabolism and cholesterol transport, making the receptor an interesting target for the potential treatment of atherosclerosis.

The nucleotide-like orphan receptor GPR17, whose endogenous agonist remains still unknown, is also G_i-coupled. GPR17 appears to be involved in demyelinating diseases such as multiple sclerosis, and some inflammatory diseases.

The development of potent, selective ligands, agonists and antagonists, for both $P2Y_{13}$ and GPR17, is required to study their physiological and pathophysiological roles and for validating the receptors as future drug targets.

In the present study, we aim to find out, if the approach via β-arrestin assay employing an enzyme complementation is suitable for a closer characterization of the human $P2Y_{13}$ and the human GPR17 receptor. Moreover, we want to study whether this approach would be useful for compound screening, identifying activators or inhibitors of each of those two receptors.

The human GPR17 containing a β-galactosidase fragment was expressed in Chinese hamster ovary cells (CHO) that express β-arrestin containing the second complementary fragment of β-galactosidase (obtained from DiscoveRx). This recombinant cell line is used for test compound screening. GPR17 activation leads to β-arrestin recruitment which results in enzyme complementation and allows β-galactosidase-dependent chemiluminiscence measurement.

Our goal is to screen for agonists and antagonists of GPR17 and to obtain an analogous cell line for the human $P2Y_{13}$ receptor, which will allow the determination of $P2Y_{13}$ receptor activation by measuring luminescence. A β-arrestin assay will be established with an outlook to determine the potency of agonists and antagonists.

Key words: $P2Y_{13}$, GPR17, β-arrestin assay, compound screening

ABSTRAKT

Identification and characterization of G protein-coupled nucleotide and nucleotide-like receptors P2Y13 and GPR17

Purinergní receptory jsou rozděleny do třech podskupin, jimiž jsou P1(adenosinové) receptory, P2 (nukleotidové) receptory a P0 (adeninové) receptory. P2 receptory se skládají z ligandem řízených iontových kanálů (LGICs) označených jako receptory $P2X_{1-7}$ a z receptorů nazývajících se $P2Y_{1,2,3,6,12-14}$, náležících do skupiny receptorů spřažených s G proteinem, a to do třídy A a její větve značené δ. Receptor $P2Y_{13}$ je fyziologicky aktivován přes ADP a jeho aktivací se spouští inhibice adenylatcyklasy (G_i). Svou stimulací vykazuje receptor $P2Y_{13}$ neuroprotektivní účinky. Dále hraje roli též v metabolismu lipoproteinů a transportu cholesterolu v těle. Receptor $P2Y_{13}$ by tedy mohl být i zajímavým terapeutickým cílem při léčbě atherosklerozy.

Receptor GPR17, podobný výše zmíněným nukleotidovým receptorům (nucleotide-like receptor) náleží k početné skupině tzv. orphan receptors, které stále postrádají svého endogenního agonistu. GPR17 je také spřažen s G proteinem (G_i) a jsou mu přisuzovány role v demyelinizačních onemocněních jako je například roztroušená skleroza nebo v některých zánětlivých onemocněních.

Vývoj silných selektivních ligandů, agonistů i antagonistů, pro receptor $P2Y_{13}$ i pro receptor GPR17 je nutný ke studiu fyziologických a patofyziologických rolí obou zmíněných receptorů a k jejich validaci jako možných terapeutických cílů.

V této práci chceme zjistit, zdali by přístup využívající komplementaci enzymu za použití metody β-arrestin assay mohl být vhodný pro bližší charakterizaci obou zmíněných receptorů, lidského $P2Y_{13}$ a lidského GPR17. Chceme též zkoumat, zdali tento přístup bude užitečný při screeningu chemických látek a jejich identifikaci jako možných agonistů nebo antagonistů obou těchto receptorů.

Lidský GPR17 receptor obsahující část enzymu β-galaktosidasy je exprimován v buňkách ovárií čínského křečka (CHO). Tyto buňky tvoří protein β-arrestin nesoucí komplementární část β-galaktosidasy. Tato připravená rekombinantní buněčná linie bude využita ke screeningu chemických látek. Aktivace receptoru GPR17 vede k rekrutaci β-arrestinu a jeho vazbě na receptor, přičemž dochází ke komplementaci obou částí β-galaktosidasy ve funkční enzym. Díky tomu jsou umožněna měření chemiluminiscence závislé na β-galaktosidase.

Naším cílem je testovat chemické látky jako potenciální agonisty či antagonisty na receptoru GPR17, získat analogickou buněčnou linii i pro receptor $P2Y_{13}$ umožňující určení aktivace receptoru díky měření luminiscence, a ustavit tak metodu pro zjišťování potencí agonistů a antagonistů.

Klíčová slova: $P2Y_{13}$, GPR17, β-arrestin assay, compound screening

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1 Introduction

1.1 Basic overview of GPCRs

The G protein-coupled receptors (GPCRs) are trans-membrane proteins with an ability of responding to a wide span of chemical stimuli in very selective manner, converting emerged signals into intracellular space and launching the intracellular signaling pathways. The GPCRs create the biggest human receptor family which is currently known.[1] More than 800 sequences were found in the human genome, encoding the GPCRs.[1] Such a plentiful occurrence and high abundance throughout various tissues of the human body indicate essential importance of GPCRs in physiological as well as in pathophysiological processes. Nowadays, only a small fraction of around 30 of all GPCRs is a target to some marketed drug.[2] On the other hand, around 30 % drugs on the market bind their therapeutic effects with agonizing or antagonizing some GPCR.[2] From allergic rhinitis to asthma, from schizophrenia to hypertension - the drugs for treating these conditions or diseases aim GPCRs.[2] And these shining examples create still only a tiny segment in the wide palette of GPCRs affecting remedies. The GPCRs are in focus when it comes to new drugs development and we can almost surely await more drugs from this field in the future.

Speaking about stimuli, to which a GPCR can respond, there is an entire miscellaneous spectrum. From such small particles like photons or ions, via organic compounds like amines, purines or lipids to really complex substances like chemokines or hormones.[1]

1.2 Classification, architecture and structure of GPCRs

With regards to resemblance of helical chains in the trans-membrane region, mainly amino acids sequence similarity, the GPCRs are further subdivided. The older division in the six classes (class A, B, C, D, E and F) is gradually replaced by a newer, so called GRAFS division into five families.[3] The extent, to which both classification systems overlap each other, is very various. Rhodopsin family includes the former class A and with its 701 members covers a vast majority.[3] Secretin family contains receptors in the former class B and currently has 15 members.[3] Adhesion family with 24 members, glutamate family with 15 members covering the former class C and finally the frizzled/taste family with 24 members, formerly referred to as class F, make up the rest.[3] With an outlook for further scientific discoveries, these numbers are certainly not ultimate.

In general, GPCRs make up very diverse group with regards to both architecture and function. Despite such wide variability throughout the entire GPCRs group, there can be traced down some basic characteristics they all have in common. GPCRs are sometimes synonymously called seven-trans-membrane receptors (7-TM).[1] This refers to the typical architecture of the protein. The 7 trans-membrane helixes constitute a bunch which is connected together by 3 extracellular (ECL1-3) and 3 intracellular loops (ICL1-3), N-terminus of the chain being positioned in the extracellular space and C-terminus occurring inside the cell.[1] The extracellular part is the place which mainly modifies access to the binding sites in trans-membrane region and secondary structures of particular receptors vary there quite significantly.[1] This stands mainly for the second extracellular loop which is also the longest

one there.[1] Most of GPCRs do have also 2 cystein residues able to form disulfide bridge between the first and the second extracellular loop, thus resulting in various access parameters as well as in supporting the chain stability.[1] Cavities and pockets in the trans-membrane region contain ligand binding sites. Properties like shape, depth, size or electrostatic interactions can vary greatly even between the same subfamily members or for receptors with same endogenous ligand.[1] The trans-membrane region serves also as signal transducer.[1] Transferring extracellular stimulus into intracellular space depends on the conformational changes of the protein.[1] Finally, the downstream signaling pathways are triggered by interactions between intracellular part of receptor and G proteins, G protein-coupled receptor kinases, arrestins or other in the cytosol present proteins.[1]

1.3 GPCRs dimers and oligomers

Receptors are mainly perceived in the basic concept of one individual protein. In the reality, they often create supramolecular structures known as dimers or oligomers.[4] These are divided according to nature of the structural units into homomers and heteromers, the former being assembled from identical units, the latter being assembled from non-identical units.[4] As the 7-TM structure of GPCR is already complex and enable a range of conformational changes, it was thought for a long time that GPCRs do not dimerize.[4] Moreover, a plenty of known GPCRs transduces stimuli from extracellular space just in the state of monomers.[4] Nevertheless, they have been already found some GPCRs (e.g. $GABA_B$ receptor) that create such supramolecular structures.[4] It is proposed that dimerization or even oligomerization can alter ligand recognition, actual receptor activation and transduction of signal or other receptor properties.[1,4] Many relations are still unclear and in order to obtain truly relevant knowledge and comprehension, more research in the field is needed.[1]

1.4 The GPCR activation and signaling

The main attribute responsible for activity of GPCRs is the G protein itself. It consists of three uneven subunits called Gα, Gβ and Gγ.[5] The conformational changes in the trans-membrane region following the receptor activation mediate exchange of GDP to GTP on the Gα subunit.[6] Gα is then broken away from other two subunits which remain together as Gβγ, as these are biologically inseparable.[6] Yet both Gα and Gβγ are able to trigger a number of intracellular pathways, thus promoting the actual effect of the receptor activation.[6] There are around 20 Gα, 6 Gβ and 11 Gγ subunits creating the immense number of heterotrimeric G proteins combinations.[5] However, speaking about the division, the character, mainly sequence similarity of the Gα subunit and follow-up downstream pathway, determines allegiance to the specific G protein group.[5,6] The four groups are currently distinguished, $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/0}$ and $G\alpha_{12/13}$.[5,6]

The $G\alpha_s$ subunit is typically responsible for activation of adenylatecyclase (AC), thus promoting transformation of intracellular ATP into cAMP.[6] A wide variety of signal transducers, transcription factors, enzymes and even ion channels plays role in an increased cAMP downstream pathway.[5] Protein kinase A (PKA) is cardinal, though not the only one point, through which majority of those pathways runs.[5]

Following the activation of $G\alpha_i$ subunit, adenylatecyclase (AC) is inhibited and the level of new synthesized cAMP in the cell decreases.[6] The $G\alpha_i$ works there together with $G\beta\gamma$, which has for example an influence on phospholipase C (PLC-β), ion channels and actually even on the AC.[5]

The $G\alpha_{q/11}$ subunit activates in the first step PLC-β, which results in inositol triphosphate (IP_3) and diacylglycerol (DAG) production.[5] The former consequently increase intracellular calcium, the latter recruits and activate protein kinase C (PKC) and its following downstream.[5]

The last from these subunits, $G\alpha_{12/13}$, influences the GTPase Rho. Nevertheless, its downstream is explored only to a limited extent and thorough mechanisms, by which the pathways work, remain still unclear.[5]

The $G\beta\gamma$ heterodimer, which breaks away from $G\alpha$ subunit upon the receptor activation and GDP-GTP exchange, plays also an appreciable role in signaling and final effect of the receptor.[7] Currently, the Kir3 channels, voltage-dependent Ca^{2+} channels, various isoforms of adenylatecyclase (AC) as well as of phospholipase C (PLC), phosphoinositide 3 kinases (PI3K) or mitogen-activated protein kinases (MAPK) are known to be regulated by $G\beta\gamma$ heterodimer.[7] Further roles are also presumed and investigated. The various combinations of different Gβ and Gγ isoforms in the final heterodimer may promote miscellaneous level of effector stimulation, making the signaling patterns really complex.[7]

1.5 GPCR allostery

Every active receptor possess binding site for its endogenous ligands, which is commonly reffered to as orthosteric binding site. However, there are also other ligands named allosteric modulators, which bind the so called allosteric binding sites. Simple ions, lipids or sterols often play this role.[1] Interaction of those substances with the receptor alters its conformation and can modulate the effect of orthosteric ligands as well as intracellular signal transducing molecules - in the case of GPCR mainly G proteins or β-arrestins.[1,8] The latter means that even certain pathways can be promoted or excluded upon allosteric modulators activity.[8] Currently, allosteric modulators are known in all receptor superfamilies, GPCRs being thus no exception.[8] According to effect which these substances show in relation to orthosteric ligands, the division into positive or negative allosteric modulators (PAM/NAM) and neutral allosteric ligands (NAL) has been implemented.[8] Yet the reality is more complex. It always depends on orthosteric ligand or pathway which is investigated, thus for example, one compound can be PAM in relation to one orthosteric ligand and NAM or NAL to another.[8] There even exist substances which are agonists to receptor and PAM at once.[8] Other important concepts of allosteric modulators are selectivity, ceiling limit and probe dependence.[8] Allosteric sites can be more diverse than the orthosteric sites with regards to subtypes sequence similarity.[8] Because of this sequence variability and altered parameters, the needed selectivity across the subtypes can be achieved.[8] The ceiling limit means that allosteric effect of an allosteric modulator reach in some point the highest possible level.[8] It depends on maximal feasible cooperation of orthosteric and allosteric site, irrespective of modulator dosage.[8] Finally, concept of probe dependence says that the effect of allosteric modulator on the receptor

depends also on specific orthosteric ligand, which binds the mentioned receptor.[8] Drugs affecting allosteric sites are presumed to have less overdose adverse effects and to be more selective, thus making this direction in research attractive as well.[1]

1.6 β-arrestin roles in GPCRs function

The β-arrestins are intracellular proteins which exist in four isoforms - β-arrestin 1 (arrestin-2), β-arrestin 2 (arrestin-3) and two isoforms of visual arrestins in rod cells (arrestin-1) and cone cells (arrestin-4) of visual system.[9,10] Upon the discovery of β-arrestin 1 (originally 48 kDa protein), it was thought that these proteins lack function or that their function is insignificant.[10] However, a set of new discoveries in recent years revealed some more information and casted a light to issue comprehension. Both β-arrestin isoforms are ubiquitously expressed throughout the tissues and have fundamental influence on GPCRs desensitization, internalization and even signaling.[10]

The agonist-activated GPCR activates not only its effector pathways, but also via certain ways initiates its own desensitization, in order to attenuate second messenger signaling.[11] Desensitization of GPCRs can then work in two ways - homologous and heterologous.[12] The homologous desensitization means that particular subtype of GPCR loses response for a specific agonist acting on it.[12] The heterologous desensitization is more complex as response for an agonist is lost across different GPCR subtypes even when these are not activated.[12] G protein-coupled receptor kinases (GRK) are responsible for phosphorylating the activated receptor which is then labeled for β-arrestins that uncouple the GPCR from the G protein, thus switching its function effectively off.[11,12] GRKs are able to phosphorylate serine or threonine residues of ICL3 or C-terminus on solely agonist-activated GPCRs so it means that they mediate only homologous desensitization.[12] Other kinases, such as second messenger-dependent protein kinases like PKA and PKC, can in some cases introduce β-arrestin mediated desensitization as well or even induct GRK-arrestin independent desensitization.[12] More important fact is actually their ability to phosphorylate agonist-unoccupied GPCRs, which is prerequisite for heterologous desensitization.[11,12]

Receptor internalization is process closely associated with desensitization. Currently, several mechanisms are known such as internalization via clathrin-coated vesicles, caveolae or via uncoated vesicles, whereby β-arrestins play an important role in the first mentioned way, as they possess high affinity to the clathrin heavy chain and react with them.[11] The specific Leu-Xaa-Glu/Asp sequence of β-arrestin C-terminal area is essential to clathrin binding.[11] Furthermore, β-arrestins react also with the β_2 subunit of adapter protein 2 (AP2) – protein, which is vital in creating clathrin lattices, thus effectively linking them together in a complex and making them work properly.[9,11] Further progress in intracellular trafficking differs though, dividing GPCRs into two classes.[11] Some receptors (e.g. β_2 adrenergic receptor, α_{1b} adrenergic receptor, μ opioid receptor or dopamine D_{1A} receptor) prefer coupling to β-arrestin 2 over the other isoform.[11] These are called class A (do not interchange with GPCR classification).[11] The β-arrestin binding to GPCR sets in quickly after receptor activation, holds then only a short time until the receptor ends up in the clathrin coated vesicle.[11] As a result, no complexes of internalized receptor and β-arrestin are found in endosomes.[11] Class A

GPCRs show also more rapid recycling and follow-up resensitization than class B GPCRs do in the case, that the GPCR is dephosphorylated.[11] Class B GPCRs show no noticeable difference in affinity between both β-arrestin isoforms and can bind also to visual arrestins.[11] Upon the receptor internalization in the clathrin coated vesicle, the complex of GPCR and β-arrestin remains still together and can be co-localized in endosomes.[11] Prospective GPCR dephosphorylation as well as recycling and resensitization are much slower than in the case of class A.[11] V_2 vasopresine receptor, AT_{1A} angiotensin receptor, neurotensin 1 receptor or thyrotropin-releasing hormone receptor are some examples of the class B GPCRs.[11] The allegiance to class A or B depends on sequence in C-terminus area.[11]

When arrestin protein family has been found, little to no function was assigned to them. In short time, their role in desensitization as well as in internalization was discovered, lending them great importance in these processes. Further observations showed, that list of their functions was still incomplete, as β-arrestins are able to initiate signals even without GPCR activation, thus launching GPCR independent pathways.[13] They are already known to play role in an extracellular signal-regulated kinase activation (ERK1/2) via angiotensin II AT_1 receptor and β-arrestin biased agonists are presumed to have effect in cardiovascular diseases therapy, mainly heart failure.[13] It is an interesting finding that localization of GPCR-activated ERK1/2 and β-arrestin 2 activated ERK1/2 pathway significantly differs as well as the final effects.[13] RhoA/ROCK pathway is another signaling cascade which is renowned to share a relation with β-arrestins, more concretely with β-arrestin 1 isoform.[13] These pathways regulate activation of each other reciprocally.[13] As both cascades play the role in attenuating of cellular stress, they show promising outlook for therapy development.[13] Carvedilol, an already registered beta blocker, is perceived as a β-arrestin biased agonist for ERK1/2 pathway, thus exhibiting its traditional cardioprotective effect in chronic heart failure therapy.[13] However, there is a wide spectrum of other kinases, where β-arrestins act for example as adapters (tyrosine kinases of Src-family) or as scaffolds for more complex structures, activating their respective pathways (p38 mitogen-activated protein kinase complexes-p38 MAPK, c-Jun N-terminal kinase-JNK, some other ERKs.[11]

1.7 Orphan GPCRs

Up to 800 GPCRs are currently known.[14] Yet more than 100 of them still lack an endogenous ligand and are referred to as orphan GPCRs.[14,15,16] Not having obtained such elementary characteristics about them, only limited information about their pharmacology is known. However, GPCRs are an important target for new drugs development and deeper knowledge of these orphan receptors could potentially lead to a handful of new medicines, making their study an important task for drug researchers.[15,16]

In order to make some use of one particular orphan GPCR, it has to be firstly deorphanized.[14] A number of approaches have been developed so that the assignment of getting a selective ligand can be fulfilled.[14] There is a possibility of comparing sequence of an orphan receptor with a sequence of an already known receptor, trying to gain a subset of possible ligands according to their mutual similarity.[14,16] Another way lies in determining of the association between orphan receptor expression profile and the presumed ligand.[14] Sometimes, extract

from tissues is prepared and its components are purified, assuming the ligand is part of the mixture.[14,15,16] A following screening then shows whether it contains the active substance.[14,16] Reverse pharmacology is also a suitable method for GPCR deorphanizing.[14,15,16] In the first step, a DNA carrying the receptor sequence is transfected into eukaryotic cell line.[14,15,16] Afterwards, the expressed receptor is coupled to ligands and their affinities are specified.[14,15,16] The method unconditionally requires receptor expression in a satisfactory amount, quality and pure ligands and last but not least, a robust screening assay, which should be simple enough for large-scale screening.[14,16] Recently, knockout mice approach has been developed as a clever tool, showing possible functions of the receptor, indicating also physiological or phatophysiological processes alongside with potential pathways.[14] Optimalization of processes leading to deorphanization of one particular GPCR is indeed an endeavour full of challenges. It is sometimes like blind-shooting as the knowledge about the receptors are very limited, their functions are blurred and the relevant pathway activation must not always be subject only to respective GPCR, but to some other accessory proteins or specific conditions as well.[14,16]

1.8 $P2Y_{13}$ receptor

The purinergic receptors form a big group, which is further divided into three subfamilies, P1 (adenosine), P2 (nucleotide) and recently P0 (adenine) receptors.[17,18,19,20,21] P2 receptors comprise ligand-gated ion channels, $P2X_{1\text{-}7}$, and rhodopsin family (class A), δ-branch GPCRs termed $P2Y_{1,2,4,6,11,12\text{-}14}$.[3,18,19,22] Although all these P2Y receptors are activated by nucleotides, they do not constitute any homogenous group.[20] With regards to $P2Y_{13}$, it is a G_i-coupled receptor, ADP being the endogenous ligand.[20,22,23] Upon certain conditions, it can couple also G_s, thus activating its respective pathway.[22]

As far as the structure is concerned, P2Y receptors show characteristic features of GPCRs like 7-TM regions linked to each other via 3 ECLs and 3 ICLs.[22,23] Human P2Y receptors possess from 328 to 377 amino acids residues, sharing moreover from around 20 up to 50 % homology.[22] They are rich with regards to amino acid composition diversity but four cysteine residues are present in the extracellular domains of every P2Y receptor, giving possibility of disulfide bridge formation - the disulfide bridge between ECL1 and ECL2, typical for GPCRs, is supplemented by another between N-terminal domain and ECL3.[23] Only two crystal structures in the group have been resolved yet, namely $P2Y_1$ and $P2Y_{12}$.[23] The inactive state structure model of $P2Y_1$ with its antagonist indicates two different ligand-binding sites, one for nucleotide antagonists in the 7-TM and one for allosteric modulators on the interface with the cell membrane at the extracellular side.[23] The $P2Y_{12}$ structure model with an antagonist shows interesting features as well.[23] Upon antagonist binding, the disulfide bridge between ECL1 and ECL2 is broken so that cysteine residues are free to interact with active metabolites of the ligand, denoting such big rearrangements in respective domains during the process.[23] On the other hand, the $P2Y_{12}$ structure model with an agonist in an inactive state shows no such thing, binding the agonist in 7-TM region with only minor changes in receptor overall architecture.[23] $P2Y_{13}$ shares about 50 % identity to $P2Y_{12}$, having the same endogenous ligand as well.[22,23] However, there are no similar models to $P2Y_{13}$ and the previously mentioned features are simply a fact, which is worth of taking into consideration, when trying to

characterize it and certain attributes can differ. Some P2Y receptors are also known to form dimers, both hetero- or homodimers, but no such thing has been observed by $P2Y_{13}$ yet.[23]

With regards to tissue distribution, $P2Y_{13}$ is like its counterparts from P2Y receptor group quiet widespread in the brain and central nervous system, then in spleen, liver, lymph nodes, bone marrow, pancreas, heart and erythrocytes.[19,23] With the high occurrence in central nervous system, there obviously emerges a question of its effect. The suggestions exist that $P2Y_{13}$ promotes cell survival, thus having neuroprotective effect. The ERK1/2 signalization in granule neurons upon receptor activation leads to launch of transcription factors responsible for trigerring survival promoting genes like in this example CREB.[24] In some other central nervous system neuronal populations, the role in neuronal differentiation and axonal elongation is assumed.[24] Interestingly, an inverted situation is to be seen in pancreatic β-cells and enteric nervous system, where $P2Y_{13}$ deactivation triggers protective effect.[24] $P2Y_{13}$ also plays role in lipoprotein metabolism and cholesterol transport, making it potentially an interesting target for treatment of atherosclerosis.[25]

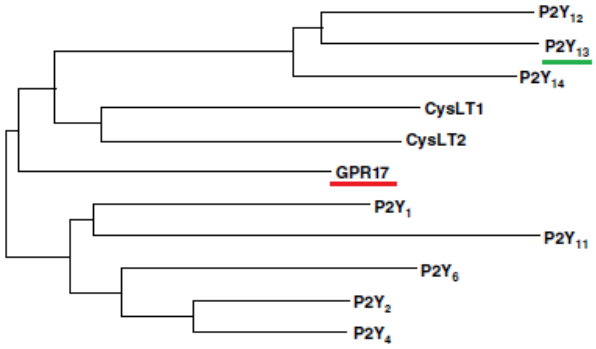
The wider application in the field of drug research is hindered by lack of highly selective agonists and antagonists. 2-MeS-ADP is useful agonist for experimental use, being more potent than endogenous agonist ADP.[20,26] However, it is not a drug-like compound. The triphosphate analogs ATP and 2-MeS-ATP then seem to act as partial agonist.[26] The numbers are more abundant with regards to antagonist-like compounds. MRS2211, a 2-chloro 5-nitro PPADS derivate, is the only one known selective antagonist on $P2Y_{13}$ yet.[20,23] Suramin, reactive blue 2, PPADS and some of its other derivates fall into group of non-selective antagonists of $P2Y_{13}$.[20,23,26] Last but not least, clopidogrel metabolite cangrelor is potent antagonist on $P2Y_{13}$, though being non-selective as well.[23,26]

For the time being, widely used $P2Y_{12}$ receptor antagonizing thienopyridine derivates clopidogrel and prasugrel, together with ticagrelor are currently registered as antiplatelet drugs, $P2Y_{2/4}$ agonist diquafosol is registered for dry eye treatment in Japan.[20] Though not numerous yet, it is probable that this group of P2Y receptors affecting drugs will grow bigger in the coming years.

1.9 GPR17 receptor

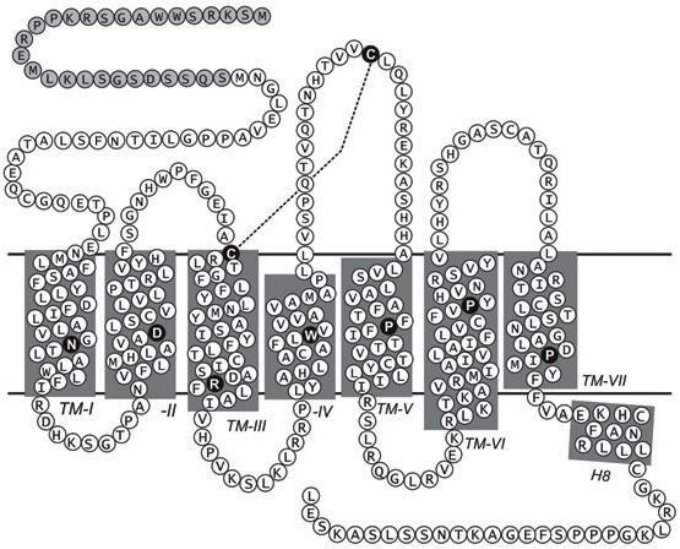
GPR17 is a rhodopsin family (class A) orphan receptor phylogenetically located between P2Y and cysteinyl leukotriene receptors (CysLTRs) (Fig. 1).[27,28,29] It is most likely G_i-coupled receptor.[28,30] However, the situation is more complicated with regards to search for its endogenous ligand. Firstly, the research group around Ciana tried to deorphanize the receptor, having claimed that GPR17 is activated by uracil nucleotides (UDP), its nucleotide sugars (UDP-galactose and UDP-glucose) and cysteinyl leukotrienes (LTC_4 and LTD_4).[28] Benned-Jensen with Rosenkilde confirmed GPR17 stimulation by UDP, UDP-galactose and UDP-glucose but were not able to reassert the same for cysteinyl leukotrienes.[30] Finally, the group around Maekawa as well as other groups did not prove that any of these ligands is activating GPR17.[31] Thus the clear identity of its endogenous ligand remains still rather ambiguous.

Figure 1 Phylogenetic tree of GPR17. The phylogenetical relations between nucleotide, nucleotide-like GPR17 receptor and CysLTRs are depicted as from the original deorphanizing report (modified from Ciana et al. 2006).[28]



Speaking about GPR17 structure, the receptor has two isoforms - the shorter consisting of 339 amino acids and the longer, possessing 28 additional amino acid residues in the N-terminus, having then 367 amino acids in total.[29,30,32] The receptor possesses 7-TM region and disulfide bond in extracellular space characteristic for GPCRs, in this case between cysteine residues of ECL1-TM III interface and ECL2 (Fig. 2).[30] The group around Parravicini nad Ranghino proposed an overview on GPR17 short isoform ligand binding features compared to those of P2Y receptors using molecular modeling, simulating the molecular dynamics as well.[29] However, as it is the only existing suggestion of the GPR 17 receptor architecture yet, more information about the GPR 17 structure using other methods would be indeed of significant importance.

Figure 2 Primary structure of human GPR17 receptor. The 7-TM region characteristic for GPCRs is preserved as well as disulfide bond between cysteinyl residues in extracellular space. The amino acids residues typical for rhodopsin-like GPCRs, which are highly conserved throughout the group, are marked as black circles. The N-terminus of the longer isoform containing 28 additional amino acids residues is marked as grey circles (modified from Benned-Jensen et al. 2010).[30]



Both GPR17 isoforms are primarily expressed in central nervous system, showing incredibly similar expression patterns from thalamus, where the expressions are the lowest, progressively through hypothalamus, cerebellum, amygdale, cerebellar hemispheres, frontal cortex to hippocampus and putamen, where the expressions are the highest.[28,30] The ratio between both isoforms is not equal though, short form being expressed in around 10-fold higher number in the most cases.[30] GPR17 occurs in heart and kidneys as well, though in significantly lower numbers.[28,30] Longer isoform is prevalent in these tissues, being the only present isoform in kidneys and having about 12-fold higher excess in heart over the shorter one.[30] The longer isoform is more abundant in those organs even in comparison to its total brain level, 7-fold higher number for heart and 2-fold higher for kidneys respectively.[30] Some low quantity occurs in the liver and lungs as well.[28] It is an interesting fact that the highest expression is observed in organs able of undergoing ischemic damage.[28] On the cellular level, study of Ciana and his colleagues shows that neuronal expression of GPR17 in brain is clearly higher in span of hours after ischemia, GPR17 being highly expressed in pyramidal neurons.[28] This research group also suggested that receptor may be expressed only in specific neuronal types.[28] The supporting astrocytes do not express GPR17 according to their results.[28] However, there are ambiguities as well, because Chen et al. 2009 suggested that GPR17 in mouse is primarily, if not only, expressed in oligodenrocytes mainly during first postnatal weeks with following decrease in subsequent weeks.[33] The physiological role of GPR17 is still blurred. Nevertheless, some roles in pathophysiological processes became successively revealed. GPR17 levels are increased in the neurons after an ischemia and ischemia-followed reperfusion, both in the ischemic core and also in boundary zone, though with different profiles, most likely mediating and propagating the injury.[28,34,35] In fact, due to implication that the GPR17 inhibition *in vivo* is reported to attenuate ischemic damage, actually even after ischemia onset.[28,34,35] It was also reported that GPR17 could play crucial role in demyelinization diseases as the receptor up-regulation is observed in demyelinating lesions (e.g. human MS plaques) and GPR17 overexpressing mice oligodendrocytes show inhibited myelin sheath formation or reactive gliosis - signs typical for oncoming demyelinization disease.[33] There are also indications, that GPR17 influences oligodendrocyte differentiation as its negative regulator.[33,36,37] Nevertheless, current knowledge is not restricted to central nervous system only. GPR17 in mice plays role in immune system, as its deficiency increases pulmonary inflammation mediated via cysteinyl-leukotrienes $CysLT_1$ receptor induced by house dust mite *Dermatophagoides farinae* (Df), connected with significant elevation of neutrophils, eosinophils and lymphocytes levels in lungs together with increase of total IgE, Df-specific IgG1 as well as with promoted Th2/Th17 cytokine expression. GPR17 works there for $CysLT_1$ receptor as its negative, ligand-independent regulator.[38]

Although identity of GPR17 endogenous ligand remains still unclear, there have been already discovered compounds affecting the receptor. Two synthetically prepared small-molecule compounds, originally intended as NMDA receptor antagonists, were tested by various functional assays (G_q-mediated intracellular calcium mobilization, G_i-coupled AC inhibition, G_s-coupled AC stimulation and dynamic mass redistribution) in multiple cell types and identified as GPR17 agonists: MDL29,951 (referred to as RA-II-150 in the patent US20130059899 A1) and GV150526A (gavestinel).[36,39] The former compound was

characterized to be selective to GPR17, activating neither P2Y nor CysLT receptors, making the compound an important instrument in following GPR17 screening.[36,39] RA-II-150 is also becoming a lead compound for synthesis and characterization of novel synthetically prepared agonists. With regards to antagonist-like acting compounds, there are no selective antagonists identified yet. Ciana et al. 2006 claim, that cangrelor ($P2Y_{12}$ and $P2Y_{13}$ antagonist), MRS2179 ($P2Y_1$ antagonist) and $CysLT_1$ receptor antagonists montelukast and pranlukast work as GPR17 antagonist as well.[28] Hennen et al. 2014 and Köse et al. 2014 focused themselves on these CysLT1 antagonists, the former stating that only pranlukast shows antagonist-like effect, the latter confirming both as antagonists.[36,39] This can be possibly caused by the compound promiscuity in the functional assays, when interaction of those compounds with natively expressed receptors may offset the effect arranged by GPR17.[39] The issues in the field of GPR17 synthetically prepared ligands remain still open for further research, which could reveal more information and clarify all current ambiguities.

1.10 Intent of the presented work

In the present study, we aim to find out, if the approach via β-arrestin assay employing an enzyme complementation is suitable for a closer characterization of the human $P2Y_{13}$ and the human GPR17 receptor. Moreover, we want to study whether this approach would be useful for compound screening, identifying activators or inhibitors of each of those two receptors. The human GPR17 containing a β-galactosidase fragment was expressed in Chinese hamster ovary cells (CHO) that express β-arrestin containing the second complementary fragment of β-galactosidase (obtained from DiscoveRx). This recombinant cell line is used for test compound screening. GPR17 activation leads to β-arrestin recruitment which results in enzyme complementation and allows β-galactosidase-dependent chemiluminiscence measurement. Our goal is to screen for agonists and antagonists of GPR17 and to obtain an analogous cell line for the human $P2Y_{13}$ receptor, which will allow the determination of $P2Y_{13}$ receptor activation by measuring luminescence. A β-arrestin assay will be established with an outlook to determine the potency of agonists and antagonists.

2 Materials

2.1 Water purification

The demineralized water for solution preparations was obtained by Milli-Q® Water System, Millipore Eschborn). For molecular biology, sterile UltraPure™, Gibco distilled water was used (further referred to as PCR water).

2.2 Sterilization

Equipment, instruments, media, solutions or other materials able to withstand the conditions of autoclaving (121 °C, $1.2x10^5$ Pa, 21 min) were autoclaved in Systec VX-95, Systec. The warm-instable solutions were sterilized in the safety cabinet using sterifilter with 0.2 μm pores (Filtropur S 0.2, Sarstedt).

2.3 Chemicals

The chemicals which were utilized for the purposes of the presented work are clearly depicted in the Tab. 1.

Table 1 Substances used in the preparations

Chemicals	**Article number**	**Producer**
Dimethyl sulphoxide, DMSO	4720.3	Roth
Dimethyl sulphoxide, DMSO (for cell culture)	A994.2	Roth
Dipotassium hydrogen phosphate anhydrous, K_2HPO_4	6875.2	Roth
Potassium dihydrogen phosphate, KH_2PO_4	P018.1	Roth
Sodium chloride, NaCl	9265.1	Roth
Magnesium acetate tetrahydrate, $Mg(CH_3COO)_2.4H_2O$	0275.1	Roth
3-((3 Cholamidopropyl)dimethylammonium)-1-propanesulfonate, CHAPS	FC12196	Carbosynth
Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA-$Na_2.2H_2O$	E5134-50G	Sigma-Aldrich
Ethylenediaminetetraacetic acid disodium salt (0.5M), EDTA-Na_2. $2H_2O$	E7889	Sigma-Aldrich
D-(+)-Glucose	G-7021	Sigma-Aldrich
Kanamycin sulphate	T832.1	Roth
Glycerol 99%	158920010	Acros
Acetic acid (glacial)	1.000.631.011	Merck
Ethylenediaminetetraacetic acid, EDTA (for TAE)	8040.2	Roth
Tropix® Emerald-II™	T2115	Thermo Fisher
Tropix® Galacton-Star® Substrate	T2265	Thermo Fisher
Sodium hydroxide pelleted, NaOH	1375.1000	Th. Geyer
Hydrochloric acid 37%, HCl	4625.2	Roth
PBS (10X Dulbecco's) powder	A0965,9010	Applichem
Phenol Red Solution 0.5%	P0290	Sigma-Aldrich
Trypsin	P10-022100	Pan Biotech
LB-Medium powder	X968.2	Roth
LB Agar powder	X969.2	Roth
LE Agarose	50004L	Lonza
Lipofectamine® 2000	11668-019	Thermo Fisher
2-(methylthio)adenosine 5'-diphosphate, 2-MeS-ADP	M152	Sigma-Aldrich

2.4 Chemicals not acquired by purchase

All compounds, which were not acquired by purchase, were synthesized by AK Müller research group (further referred to as AK Müller), Pharmaceutical Chemistry, University of Bonn. RA-II-150 is a GPR17 agonist.[36,39] OLE29, S6067, Yazh 183, Yazh 664, Yazh 665, Yazh 678, Yazh 679, Yazh 690, Yazh 691, Yazh 697, Yazh 698, Yazh 703, Yazh 704, Yazh 706, Yazh 721, Yazh 722 and Yazh 723 were synthesized and tested as possible GPR17 agonists. ANM06, ANM07, ANM20, ANM21, ANM22, ANM23, ANM27, ANM34, ANM40, ANM47, ANM68, ANM77, ANM78, ANM111, ANM158, ANM182, ANM183, ANM250, ANM251, ANM252, ANM263, ANM264, ANM265, ANM267, ANM270, ANM279, ANM280, ANM287, ANM295, ANM296, ANM297, ANM298, ANM299, ANM300, ANM334, ANM335, ANM337 and ANM346 were synthesized and tested as possible GPR17 antagonists.

2.5 Chemical structures of applied compounds

The structures of these chemicals are noted in the results part (see 4.3, 4.4, 4.5) with regards to their function obtained via their screening.

2.6 Buffers and solutions

PBS buffer (10X)

The powdered PBS (10X Dulbecco's) was dissolved in 500 ml of the final volume and stirred for a while. The final volume of distilled water was then filled in, the flask autoclaved and PBS buffer (10X) was prepared (Tab. 2).

Table 2 PBS buffer (10X)

Constituent	Amount	Final concentration
PBS (10X Dulbecco's) powder	95.50 g	10X
Demineralized H_2O	ad 1000 ml	-

PBS buffer (1X)

The PBS buffer (10X) was used for the PBS buffer (1X) preparation. The appropriate volume of demineralized water has been utilized for the dilution, pH was adjusted to the value of 7.4 and the flask was autoclaved (Tab. 3). The prepared PBS buffer (1X) was stored at room temperature.

Table 3 PBS buffer (1X)

Constituent	Amount	Final concentration
PBS buffer (10X)	50 ml	1X
Demineralized H_2O	ad 500 ml	-

Dissociation buffer (dissociating the cells before seeding them for an assay)

EDTA-Na_2 was added to PBS buffer, pH was then adjusted to the value of 7.4. After autoclaving, 5 ml of sterile 1M D-(+)-Glucose was added aseptically, resulting in final 10mM concentration of D-(+)-Glucose in the prepared dissociation buffer (Tab. 4).

Table 4 Dissociation buffer

Constituent	Amount	Final concentration
EDTA-Na_2	372.24 mg	2 mM
D-(+)-Glucose (1M)	5 ml	10 mM
PBS buffer	500 ml	-

0.5M EDTA

EDTA (for TAE) was dissolved in the demineralized water. The pH was adjusted to 8.0 by NaOH, as this is pH in which EDTA dissolves completely. The volume was then filled in by

demineralized water to 1000 ml (Tab. 5). Finally, the bottle was autoclaved and stored in room temperature for the use.

Table 5 0.5 M EDTA

Constituent	Amount	Final concentration
EDTA (for TAE)	186.1 g	0.5 M
Demineralized H_2O	ad 1000 ml	-

Lysis buffer (lysis of cells before an assay)

The chemicals needed for the preparation of lysis buffer were dissolved in the appropriate amount of the solvent. Afterwards, pH was adjusted to the value of 7.5 (Tab. 6).

Table 6 Lysis buffer

Constituent	Amount	Final concentration
K_2HPO_4	0.2178 g	5 mM
KH_2PO_4	0.1703 g	5 mM
NaCl	2.1915 g	150 mM
$Mg(CH_3COO)_2.4H_2O$	0.5363 g	10 mM
CHAPS	5 g	2 %
PBS buffer	ad 250 ml	-

Tris-acetate/EDTA buffer (50X TAE)

Tris was dissolved in 50-60 % of total demineralized water volume, EDTA and glacial acetic acid were added and the volume was filled in to 1 l (Tab. 7). Final Tris-acetate/EDTA buffer (1X TAE) was prepared by appropriate dilution. The final concentrations in 1X TAE were 0.04 M of Tris-acetate and 0.001 M of EDTA.

Table 7 Tris-acetate/EDTA buffer (50X TAE)

Constituent	Amount	Final concentration
Tris	242.00 g	2 M
Glacial acetic acid	57.10 g	5.71 %
0.5 M EDTA	100.00 ml	0.05 M
Demineralized H_2O	ad 1000 ml	-

Trypsin/EDTA-Na_2 (0.05 %/0.6 mM) (TE)

The needed volume of 0.5M EDTA-Na_2 was filled in by 2 l of PBS buffer (1X) and the flask was autoclaved. Using safety cabinet, Trypsin and Phenol Red solution were added, so there was no contamination. In the end, the 50 ml aliquots were prepared and stored in refrigerator at 2-8 °C (Tab. 8).

Table 8 Trypsin/EDTA-Na_2 (0.05 %/0.6 mM)

Constituent	Amount	Final concentration
EDTA-Na_2 (0.5 M solution)	2.4 ml	0.6 mM
Trypsin (2.5 % in PBS)	40.0 ml	0.05 %
Phenol Red Solution 0.5 %	1.5 ml	0.000375 %
PBS buffer (1X)	ad 2000 ml	-

2.7 Media and solutions for cell culture

2.7.1 Media and cell supplements

The buffers, basic media solutions and supplements for cell culture work are represented in the Tab. 9. Fetal bovine serum was stored in the freezer as 50ml aliquots, Paneticin G_{418} (100 mg/ml) was stored in the freezer as 4ml aliquots, Hygromycin B Gold (100 mg/ml) was stored in the freezer as 5ml aliquots, Penicillin/Streptomycin (10000 U/ml Penicillin, 10 mg/ml Streptomycin) was stored in freezer as 5ml aliquots (Tab. 9). These all were thawed shortly before the use.

Table 9 Media and cell supplements

Name	Article number	Producer
F12 Nut Mix (Ham) (1X)	21765-029	Gibco/Invitrogen™
Fetal bovine (calf) serum	F7524	Sigma-Aldrich
Paneticin G_{418} (100 mg/ml)	P06-17200	Pan Biotech
Hygromycin B Gold (100 mg/ml)	ant-hg-5	InvivoGen
Penicillin-Streptomycin (10000 U/ml Penicillin, 10 mg/ml Streptomycin)	P06-07100	Pan Biotech
OptiMem® I (1X)	11058-021	Gibco/Invitrogen™

2.7.2 Specific cell culture media

Medium for empty CHO β-arrestin cells

The previously prepared and frozen stock solutions of constituents are thawed in a 37 °C water bath. Afterwards, the constituents are added to F12 Nutrient mixture in an aseptic environment, using working chamber with laminary air flow (Tab. 10).

Table 10 Medium for empty CHO β-arrestin cells

Constituent	Volume	Final concentration
Fetal bovine (calf) serum	50 ml	~10 %
Penicillin-Streptomycin solution	5 ml	~100 units/ml Penicillin ~0.1 mg/ml Streptomycin
Hygromycin B	4.5 ml	~900 μg/ml
F12 Nutrient mixture	500 ml	-

Antibiotic-free medium (for $P2Y_{13}$ PK2-ARMS2 transfection)

The previously prepared and frozen FCS stock is thawed in a 37 °C water bath. Afterwards, the FCS is added to F12 Nutrient mixture in an aseptic environment, using working chamber with laminary air flow (Tab. 11).

Table 11 Antibiotic free medium

Constituent	Volume	Final concentration
Fetal bovine (calf) serum	50 ml	~10 %
F12 Nutrient mixture	500 ml	-

Selection medium for $P2Y_{13}$ PK2-ARMS2 expressing CHO cells

The previously prepared and frozen stock solutions of constituents are thawed in a 37 °C water bath. Afterwards, the constituents are added to F12 Nutrient mixture in an aseptic environment, using working chamber with laminary air flow (Tab. 12).

Table 12 Selection medium for $P2Y_{13}$ PK2-ARMS2 expressing CHO cells

Constituent	Volume	Final concentration
Fetal bovine (calf) serum	50 ml	~10 %
Penicillin-Streptomycin solution	5 ml	~100 units/ml Penicillin ~0.1 mg/ml Streptomycin
Hygromycin B	4.5 ml	~900 µg/ml
Paneticin G_{418}	4.0 ml	~800 µg/ml
F12 Nutrient mixture	500 ml	-

Selection medium for GPR17-PK1 expressing CHO cells

The previously prepared and frozen stock solutions of constituents are thawed in a 37 °C water bath. Afterwards, the constituents are added to F12 Nutrient mixture in an aseptic environment, using working chamber with laminary air flow (Tab. 13).

Table 13 Selection medium for GPR17-PK1 expressing CHO cells

Constituent	Volume	Final concentration
Fetal bovine (calf) serum	50 ml	~10 %
Penicillin-Streptomycin solution	5 ml	~100 units/ml Penicillin ~0.1 mg/ml Streptomycin
Hygromycin B	1.5 ml	~300 µg/ml
Paneticin G_{418}	4.0 ml	~800 µg/ml
F12 Nutrient mixture	500 ml	-

Assay medium for GPR17-PK1 as well as for $P2Y_{13}$ PK2-ARMS2 expressing CHO cells

The previously prepared and frozen stock solutions of constituents are thawed in a 37 °C water bath. Afterwards, the constituents are added to basic OptiMem medium in an aseptic environment, using working chamber with laminary air flow (Tab. 14).

Table 14 Assay medium

Constituent	Volume	Final concentration
Fetal bovine (calf) serum	10 ml	~2 %
Penicillin-Streptomycin solution	5 ml	~100 units/ml Penicillin ~0.1 mg/ml Streptomycin
Hygromycin B	1.5 ml	~300 µg/ml
Paneticin G_{418}	4.0 ml	~800 µg/ml
OptiMem	500 ml	-

2.8 Characteristics of analyzed cell lines

The cell lines applied in the β-arrestin assay are clearly depicted in the Tab. 15.

Table 15 Analyzed cell lines characteristics

Name	Characterization	Origin
CHO $P2Y_{13}$-PK2-ARMS2	Chinese hamster ovary	Empty β-arrestin CHO cells (generously provided by A. Abdelrahman, AK Müller)
CHO GPR17-PK1	Chinese hamster ovary	Generously provided by A. Abdelrahman, AK Müller

2.9 Media and solutions for bacterial culture

2.9.1 Transformation and cultivation

Kanamycin (50 mg/ml)

Prepared solution was put through sterile filtration and stored in freezer at -20 °C in the form of 1ml aliquots (Tab. 16).

Table 16 Kanamycin (50 mg/ml)

Constituent	Amount	Final concentration
Kanamycin sulphate	1 g	50 mg/ml
Demineralized H_2O	ad 20 ml	-

Luria-Bertani medium (LB medium) antibiotic free or with 50 µg/ml kanamycin*

The LB medium was prepared by mixing 25 g of powder with appropriate amount of demineralized water and follow-up autoclaving. In the case of LB medium with antibiotic, the appropriate volume of kanamycin was added after autoclaving and cooling down (Tab. 17). Actually, the LB-medium is a mixture of nutrients, whose exact composition is not entirely known. Tryptone and yeast extract that compose the powder, provide organic compounds. The former contains particularly amino acids and short-chain peptides, the latter sugar and some other organic nutrients. Besides, sodium chloride is part of the powder mixture.

Table 17 LB medium with/without kanamycin

Constituent	Amount	Final concentration
LB-Medium powder	25 g	2.5 %
(Kanamycin 50 mg/ml)*	1 ml	50 µg/ml
Demineralized H_2O	ad 1000 ml	-

Luria-Bertani agar with 50 µg/ml kanamycin agar (LB agar with 50 µg/ml kanamycin)

Firstly, 800 ml demineralized water was added to 32 g LB Agar powder. After the agar melted using the microwave, the volume was adjusted to 1000 ml by demineralized water and the solution was autoclaved. When the solution cooled down to approximately 40 °C, kanamycin was added in the appropriate volume. The prepared 10 cm Petri dishes were then filled by 20 ml each and cooled down till they became solid (Tab. 18). The dishes were stored in plastic bag in a fridge at 4 °C.

Table 18 LB Agar with kanamycin (preparation of the dishes)

Constituent	Amount	Final concentration
LB Agar powder	32 g	3.2 %
Kanamycin (50 mg/ml)	1 ml	50 µg/ml
Demineralized H_2O	ad 1000 ml	-

2.10 Bacterial strains

The all utilized bacterial strains are represented in the Tab. 19.

Table 19 The bacterial strains

Name	Function	Origin (Catalog No.)
TOP 10 competent *E. coli*	Chemically competent *E. coli* bacteria suitable for transformation	Invitrogen (C404010)

2.11 Consumables

The consumables needed for the laboratory tasks are summarized in the list depicted in the Tab. 20.

Table 20 The list of consumables

Article	Article number	Producer
Cellstar® 6 wells cell culture plate	657160	Greiner bio-one
Cellstar® 24 wells cell culture plate	662160	Greiner bio-one
Transparent 96 wells cell culture plate	83.3924	Sarstedt
Cell culture flask (25 cm^2)	83.3910.002	Sarstedt
Cell culture flask (75 cm^2)	83.3911.002	Sarstedt
Cell culture flask (175 cm^2)	83.3912.002	Sarstedt
Culture (Petri) dishes (10 cm)	CLS430167	Sigma Aldrich
Falcon centrifuge tube (15 ml)	352096	Corning
Falcon centrifuge tube (50 ml)	352070	Corning
Cryopure tubes 1.6 ml mix color	72.380.992	Sarstedt
Reagent tube 0.5 ml Safe Seal	72.699	Sarstedt

Reagent tube 1.5 ml Safe Seal	72.706.400	Sarstedt
Reagent tube 2.0 ml Safe Seal	72.695.400	Sarstedt
Tube 13 ml	62.515.006	Sarstedt
Maxi Vial 18 ml	6000201	Perkin Elmer
5 ml serological pipette	86.1253.001	Sarstedt
10 ml serological pipette	86.1254.001	Sarstedt
25 ml serological pipette	86.1685.001	Sarstedt
200 µl graduated filter tips	S1120-8810	TipOne® Starlab
100 µl filter tips	70.760.212	Biosphere® Sarstedt
1000 µl filter tips	70. 762. 211	Biosphere® Sarstedt
10 µl graduated, extra long tips	VT0100X	Biozym
200 µl tips	70. 760. 002	Sarstedt
1000 µl graduated tips	S1111-6000	TipOne® Starlab
Parafilm™	1447011	Labomedic
PCR tubes (0.2 ml)	72.737.002	Sarstedt
Reservoir sterile, individual	89094-682	VWR
Reservoir nonsterile, bulk packed	89094-674	VWR
Nunclon™Delta Surface 96-well cell culture plate	136101	Thermo Scientific
5 ml Ritips® Professional dispenser tip	40007-0006	Ritter
V-profile micro plate	9292.1	Roth
Filtropur S 0.2	83.1826.001	Sarstedt

2.12 Molecular biology material

The enzymes, buffers, molecular weight markers and other materials for molecular biology are summarized in the most enlightened Tab. 21.

Table 21 Molecular biology material

Name	**Product number**	**Producer**
Q5® High-Fidelity (HF) DNA Polymerase (2000 U/ml)	M0491S	New England BioLabs
Q5® Reaction buffer (5X)	B9027S	New England BioLabs
Q5® High GC Enhancer (5X)	B9028A	New England BioLabs
NheI-HF® (20000 U/ml)	R3131S	New England BioLabs
HindIII-HF® (20000 U/ml)	R3104S	New England BioLabs
CutSmart® buffer (10X)	B7204S	New England BioLabs
Purified BSA (100X)	B9001S	New England BioLabs
10 mM ATP	P0756S	New England BioLabs
dNTP mixture (2.0 mM each)	R0242	Thermo Fisher Scientific
T4 DNA Ligase (5 U/µl)	EL0011	Thermo Fisher Scientific
Buffer for T4 DNA Ligase (10X)	B69	Thermo Fisher Scientific
Lambda DNA/EcoRI plus HindIII Marker (Lambda marker)	SM0191	Thermo Fisher Scientific
ΦX174 DNA-Hae III Digest (Phi marker)	N3026L	New England Biolabs
Pyrobest® DNA polymerase (5 U/µl)	R005A	Takara
Pyrobest® buffer II (10X)	R005A	Takara
Nucleic acid gel stain GelRed™ (10000X)	41003	Biotium

2.13 Kits for molecular biology

The kits utilized in molecular biology methods are denoted in the Tab. 22.

Table 22 The kits for molecular biology methods

Kit	Article number	Producer
DNA Clean & Concentrator™-5	D4014	Zymo Research
Zymoclean™ Gel DNA Recovery Kit	D4002	Zymo Research
ZR Plasmid Miniprep™-Classic	D4054	Zymo Research
PureLink™ HiPure Plasmid Filter Midiprep Kit	K2100-15	Thermo Fisher Scientific

2.14 Oligonucleotides

The oligonucleotides (primers) used in the PCR with $hP2Y_{13}$ are depicted in Tab. 23.

Table 23 Oligonucleotides

Sequence (5'-3')	Function	Producer
gtgacagctagcatgactgccgccataagaag	Forward cloning primer	Invitrogen
cttactaaagcttgcgcctaaggttatgttgtctg	Reverse cloning primer	Invitrogen

2.15 Vector plasmids

The vector plasmids which were used in the presented work are represented in the Tab. 24, the former vector was used for human $P2Y_{13}$, the latter for human GPR17.

Table 24 Vector plasmids

Vector	Resistence/selection	Size (bp)	Promoter	Producer
pCMV-ARMS2-PK2	kanamycin/neomycin	4172	CMV Promoter	DiscoveRx
pCMV-PK1	kanamycin/neomycin	4133	CMV Promoter	DiscoveRx

2.16 Laboratory instruments and equipment

The list of instruments or equipment used in the work is summarized in the Tab. 25.

Table 25 The list of instruments and equipment

Article	Article name or type	Producer
Autoclave	Systec VX-95	Systec
Balances	CPA225D	Sartorius
Balances (Isolab)	SBC42	Scaltec
Centrifuge 1 (cell culture)	ROTOFIX 32 A	Hettich
Centrifuge 2 (midi-preparation)	Allegra™ 21R	Beckman Coulter
Centrifuge 3 (molecular biology)	Mikro 200	Hettich
Centrifuge 4 (midi-preparation)	Avanti® J-20 I	Beckman
CO_2 incubator (cell cultures)	INC 246	Memmert
Counting chamber	Neubauer	Superior Marienfeld
Heater for cell plates	DTS-4	LTF Labortechnik
Heater for compound vials	MR 3001	Heidolph
Electronic pipette filler	Easypet® 3	Eppendorf
Electrophoresis power supply	Elite 300 Plus	Wealtec
Freezer (-80 °C)	Forma™ Model 906	Thermo Fisher Scientific
Instrument for gel documentation	Universal Hood II Geldoc	Biorad
Incubator (Isolab)	Thermomixer comfort	Eppendorf
Incubator (bacterial culture)	Innova 4200 Incubator shaker	New Brunswick Scientific
Cell microplate reader	Mithras LB 940	Berthold Technologies

Microscope	Axiovert 25	Zeiss
PCR cycler	T personal	Biometra
Pipettor 0.5-10 µl 8-channel	Eppendorf Xplorer®	Eppendorf
Pipettor 5-100 µl 8-channel	Eppendorf Xplorer®	Eppendorf
pH-electrode	InLab® Semi-Micro	Mettler Toledo
pH-meter	S220 SevenCompact™ pH/Ion	Mettler Toledo
Manual pipette 0.5-10 µl	Eppendorf Research® Plus	Eppendorf
Manual pipette 10-100 µl	Eppendorf Research® Plus	Eppendorf
Manual pipette 20-200 µl	Eppendorf Research® Plus	Eppendorf
Manual pipette 100-1000 µl	Eppendorf Research® Plus	Eppendorf
Multipipette (assay)	Eppendorf Multipette® Plus	Eppendorf
Vortex	IKA VG 3	IKA
Safety cabinet	NUNC® Bioflow	NUNC
Spectrophotometr (DNA conc.)	Colibri	Titertek Berthold
Water bath	VNB 14	Memmert

2.17 Software

The list of the software which was used for measuring, processing and visualization of the results is presented in the Tab. 26.

Table 26 Software

Name of software	**Producer**	**Use**
MicroWin2000	Berthold Technologies	Processing the actual measuring of the luminescence during the assay
Office Excel 2007	Microsoft	Visualization of the results from the assay via tables
Office Word	Microsoft	Writing the results
Prism® 5	GraphPad	Processing results from Excel tables into concentration-response curves
Quantity One® 4.4.0	Bio Rad	Visualization of gels from electrophoresis

3 Methods

3.1 Molecular biology methods

3.1.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is nowadays a common procedure for a quick amplification of specific DNA sequences *in vitro*. The discovery of the method by Mullis et al. 1988 and the very beginnings of application can be dated back to late 80's.[40] Yet the yield, specifity, length and other parameters, performing the actual PCR included, greatly improved when Taq DNA Polymerase has been discovered.[40,41] The enzyme was isolated from a bacterium *Thermus aquaticus*, enabled higher reaction temperatures and significantly improved reaction parameters.[40] Still, the lack of Taq DNA Polymerase proofreading activity was a major drawback.[42] Other thermo stable DNA polymerases have been found in *Thermococcus* sp. and *Pyrococcus* sp.[42] These possess 3' to 5' exonuclease-dependent proofreading activity, thus lowering the error rate by correcting the incorrectly incorporated nucleotides.[42]

For this work, Q5® High-Fidelity DNA Polymerase and Pyrobest® DNA polymerase were used, in order to multiply the template $hP2Y_{13}$ pcDNA S (Tab. 27, 28). The template sequence was provided generously by A. Abdelrahman (AK Müller) with an outlook to express $hP2Y_{13}$ receptor in Chinese hamster ovary (CHO) cells. Both above denoted polymerases do have proofreading activity. All the accomplished PCR reactions were performed on T Personal Thermocycler, Biometra.

Table 27 Protocol of PCR using Q5® HF DNA Polymerase. The $hP2Y_{13}$ pcDNA S sequence was multiplied by PCR, the final product was concentrated by DNA Clean & Concentrator™-5 kit and eluted in 10 µl PCR water. It was proceeded as written in the manufacturer's instructions, which were provided with the kit.

Components		**PCR conditions**		
Name of component	**Volume (µl)**	**Step**	**Temperature (°C)**	**Time (min)**
Template ($hP2Y_{13}$ pcDNA S)	3.0	Initial denaturation	98	4
Q5® HF DNA Polymerase	0.5	Denaturation*	98	1
		Annealing*	61	1
Q5® Reaction buffer (5X)	10.0	Extension*	72	2
dNTP mixture (2.0 mM each)	5.0	Final extension	72	10
Forward cloning primer (10 µM)	2.5	Hold	4	-
Reverse cloning primer (10 µM)	2.5	*30 cycles of Reaction time: 2 h, 41 min		
Q5® High GC Enhancer (5X)	10.0			
PCR-Water	16.5			

Table 28 Protocol of PCR using Pyrobest® DNA polymerase. The $hP2Y_{13}$ pcDNA S sequence was multiplied by PCR, the final product has been concentrated by DNA Clean & Concentrator™-5 kit and eluted in 10 µl PCR water. It was proceeded as written in the manufacturer's instructions which were provided with the kit.

Components		**PCR conditions**		
Name of component	**Volume (µl)**	**Step**	**Temperature (°C)**	**Time (min)**
Template ($hP2Y_{13}$ pcDNA S)	3.0	Initial denaturation	95	2
Pyrobest® DNA polymerase	0.5	Denaturation*	95	0:15
		Annealing*	61	0:30
Pyrobest® buffer II (10X)	2.5	Extension*	68	4
dNTP mixture (2.0 mM each)	2.5	Final extension	68	10
Forward cloning primer (10 µM)	5.0	Hold	4	-
Reverse cloning primer (10 µM)	5.0	*30 cycles of Reaction time: 2 h, 50 min		
PCR-Water	6.5			

3.1.2 Restriction reaction

3.1.2.1 Preparative restriction reaction

The amplified PCR product ($hP2Y_{13}$ pcDNA S) and vectors (ARMS1-PK2, ARMS2-PK2) underwent the restriction reaction using the NheI-HF® and HindIII-HF® restriction enzymes, in order to obtain sticky ends. The PCR product for this reaction was prepared right after PCR by DNA Clean & Concentrator™-5 kit as written in the manufacturer's instructions and eluted in 10 µl PCR water. Preparative restriction reaction components are showed in the Tab. 29.

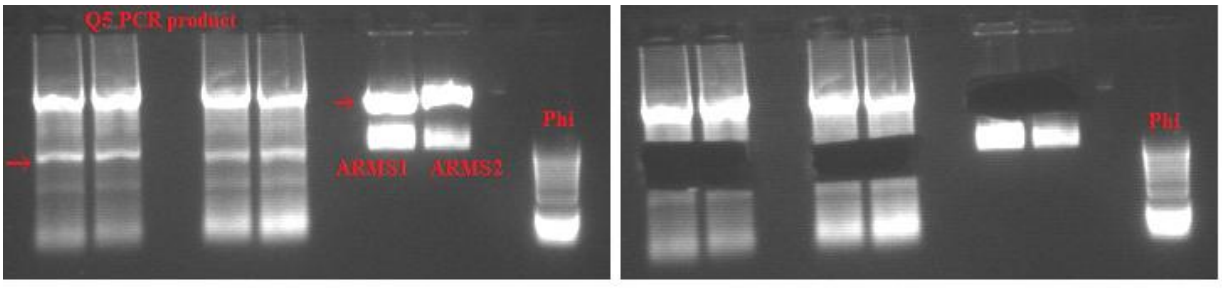
Table 29 Protocol of preparative restriction reaction

PCR product preparative restriction		Vectors preparative restriction	
Components	**Volume (µl)**	**Components**	**Volume (µl)**
PCR product (hP2Y$_{13}$ pcDNA S)	10.0	Vector	1.0
NheI-HF®	1.0	NheI-HF®	0.5
HindIII-HF®	1.0	HindIII-HF®	0.5
BSA (10X)	2.0	BSA (10X)	1.0
CutSmart® buffer (10X)	2.0	CutSmart® buffer (10X)	1.0
PCR-Water	4.0	PCR-Water	6.0

The preparative restriction reaction took 1 hour at 37 °C without any shaking program on and was performed on Thermomixer Comfort, Eppendorf. After the reaction was done, 4 µl of DNA Loading dye were added to each tube containing 20 µl of restricted PCR product total volume and 2 µl of DNA Loading dye were added to each tube containing 10 µl of restricted vector total volume. The applied DNA Loading dye volumes must always correspond to 1:6 dilution ratios. The electrophoresis was then performed on 1% agarose gel to effectively separate wanted receptor and vectors sequences in order to enable further steps. This stands for cutting corresponding parts from the gel and using Zymoclean™ Gel DNA Recovery Kit to obtain these sequences from the incised gel, proceeding as written in the instructions provided by the manufacturer. The gel slices were thawed at 55 °C for 10 minutes using Thermomixer Comfort, Eppendorf. After filtering out the impurities using columns provided with the kit, all wanted products were eluted in 10 µl PCR-Water.

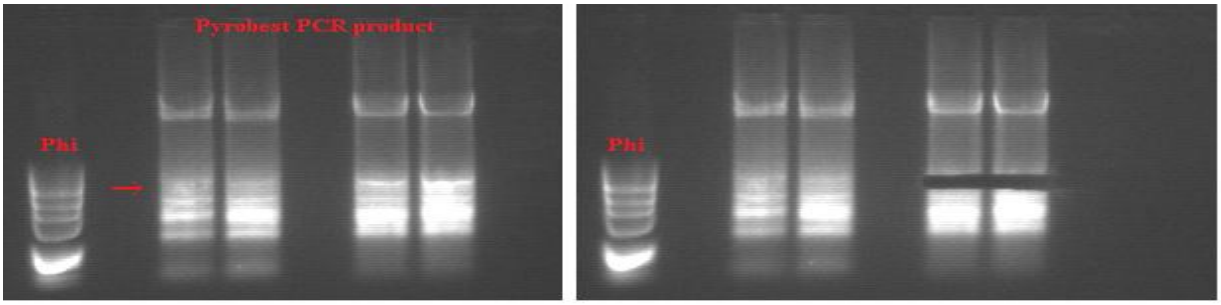
PCR product (hP2Y$_{13}$ pcDNA S) originally obtained by Q5® HF DNA Polymerase (termed as Q5 PCR product) and both vectors (shortly termed ARMS1 and ARMS2) underwent gel electrophoresis using the conditions denoted in the previous paragraph. Inserts containing expected sequence of hP2Y$_{13}$ receptor as well as both empty vectors are highlighted by red arrows in the Fig. 3. These were cut out from the gel (see the right section of the Fig. 3) for further preparations.

Figure 3 Restriction image 1



PCR product (hP2Y$_{13}$ pcDNA S) originally obtained by Pyrobest® DNA polymerase (termed as Pyrobest PCR product) underwent gel electrophoresis as well. Inserts containing expected sequence of hP2Y$_{13}$ were incised from the gel for further preparations and are marked by red arrow in the Fig. 4.

Figure 4 Restriction image 2



3.1.2.2 Analytical restriction reaction

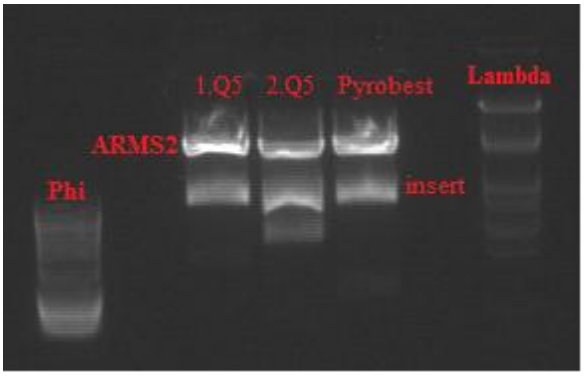
The small volumes of clean plasmid DNA were acquired by ZR Plasmid Miniprep™-Classic kit for plasmid DNA isolation. Prepared expected constructs ($hP2Y_{13}$ PK2-ARMS2) were restricted with the same restriction enzymes (NheI-HF® and HindIII-HF®) as in the preparative restriction, using Thermomixer Comfort, Eppendorf. Digestion took 95 minutes at 37 °C. The reaction components are depicted in the Tab. 30. The restricted inserts were intended for being sent to sequencing, in order to get information, whether these inserts hold the correct sequence. The electrophoresis has been performed on 1% agarose gel with certain amount of product for assurance, that the original insert can be obtained again and if so, 12 µl of product was mixed with 18 µl of PCR water, preparing the mixture for sequencing analysis.

Table 30 Analytical restriction reaction protocol

PCR product preparative restriction	
Components	**Volume (µl)**
Miniprep product ($hP2Y_{13}$ PK2-ARMS2)	5.0
NheI-HF®	0.5
HindIII-HF®	0.5
BSA (10X)	1.0
CutSmart® buffer (10X)	1.0
PCR-Water	2.0

Three tubes containing expected $hP2Y_{13}$ PK2-ARMS2 constructs, two with origin in Q5 PCR product, termed 1.Q5 and 2.Q5, one with origin in Pyrobest PCR product, termed Pyrobest (see Fig. 3, 4), were restricted for analytical purposes. Little amount of the restricted miniprep product has been taken from each tube and electrophoresis was performed. The gel was evaluated under UV light in order to determine if the original insert can be obtained again. The resulting image is represented in the Fig. 5. After the fact was confirmed that the insert could be separated and obtained again from the carrying vector, the samples were prepared for sequence analysis.

Figure 5 Electrophoresis after analytical restriction reaction



3.1.3 Ligation

T4 DNA Ligase is enzyme catalyzing the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl end in duplex DNA, joining cohesive DNA fragments with the help of its cofactor ATP together.

The $hP2Y_{13}$ DNA and PK2-ARMS2 vector with cohesive ends have been obtained by recovery from the gel after the previous preparative restriction step, mixed together in an overabundance of the $hP2Y_{13}$ DNA and incubated for 2 days at 16 °C with shaking program on, using Thermomixer Comfort, Eppendorf. Components needed for the ligation are listed in the Tab. 31. The product of reaction was used immediately for transformation on TOP10 competent *E. coli* bacteria.

Table 31 Ligation reaction protocol

PCR product – vector ligation	
Name of component	**Volume (µl)**
Template ($hP2Y_{13}$)	10.0
Vector (PK2-ARMS2)	2.0
T4 DNA Ligase	1.0
Buffer for T4 DNA Ligase	2.0
10 mM ATP	2.0
PCR-Water	3.0

3.1.4 Transformation

Transformation is method used in molecular biology for incorporation of exogenous DNA into host cells, so that these will produce this inserted DNA together with own bacterial genome. The size of insert is limiting factor for successful transformation as the bigger the inserted plasmid, the more difficult the transformation is.

For purpose of this work, transformation by the heat shock was used. The beforehand prepared aliquots of 100 µl TOP10 competent *E. coli* bacteria were thawed on ice, product from ligation reaction was added and this mixture was incubated for 30 minutes. The tube was then warmed in a 37 °C water bath for 2 minutes and laid back on the ice for other 2 minutes afterwards, causing the heat shock needed for successful procedure, making the cell

membrane permeable for to-be-inserted plasmids. Afterwards, 300 µl of antibiotic free LB medium was added to each tube and these were incubated at 37 °C, 300 RPM for an hour, using Thermomixer Comfort, Eppendorf. Following the incubation, the contents of tubes were injected on prepared 50 µg/ml kanamycin dishes, each to its own dish. These were then incubated in an inverted position overnight at 37 °C in Innova 4200 incubator, New Brunswick Scientific. On the next day, single colonies from each transformation were scratched with a tip and transferred into a separate tubes with 5 ml of selection medium (LB medium with 50 µg/ml kanamycin). The tubes were then incubated overnight at 37 °C while shaking in Innova 4200 incubator, New Brunswick Scientific. Liquid cultures were prepared for further steps.

3.1.5 Isolation of plasmid DNA

In order to obtain plasmid DNA, the kits for plasmid isolation were used, based on modified alkaline lysis.[43] The first step is basically set up on weakening the cell walls by Tris with EDTA, followed by complete lysis of the bacteria by sodium-dodecyl sulphate (SDS) with NaOH.[43] SDS serves as an anionic detergent and destroys the lipid cell membrane.[43] Both chromosomal and plasmid DNA, RNA as well as proteins get denaturated by the high pH value caused by NaOH.[43] In the second step, neutralizing potassium acetate is added.[43] The renaturated plasmid DNA stays in the solution.[43] Chromosomal DNA reaggregates into insoluble network, protein-SDS complexes with high molecular weight RNA precipitate and can be separated together with cell debris via centrifugation.[43] Plasmid DNA can be then chromatographically purified.

3.1.5.1 Analytical isolation of plasmid DNA

In order to analyze inserts in the restriction analysis, the plasmid DNA was firstly isolated in small amounts using ZR Plasmid Miniprep™-Classic kit. The colonies in 5 ml of selection medium (LB medium with 50 µg/ml kanamycin) grown from transformation were used.

It was proceeded as written in the instructions provided by the manufacturer. Bacterial culture has been taken for centrifugation in 4 ml volume, leaving 1 ml from bacterial culture for other preparations. The centrifuged bacteria were then resuspended, lysed and neutralized (see 3.1.5). After another set of centrifugation, the remnants of lysed bacteria were disposed of and the solution of wanted plasmid DNA was obtained. After all washing steps, the solution of clean plasmid DNA was acquired by elution in 30 µl of PCR water.

3.1.5.2 Preparative isolation of plasmid DNA

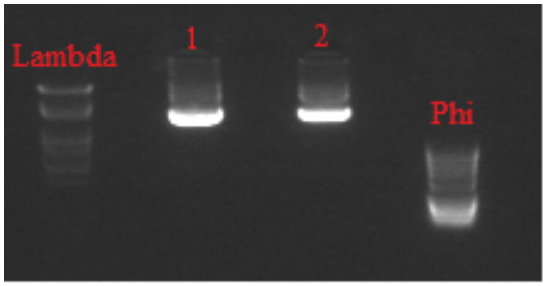
If the right sequence of insert is confirmed, it is essential to obtain sufficient amount of plasmid DNA for cell culture methods. After isolation of plasmid DNA for analysis, 1 ml of bacterial culture remained for further steps. As this was not enough for any preparation, 100 ml liquid culture has been prepared from it, using selection medium (LB medium with 50 µg/ml kanamycin). Following the overnight incubation at 37 °C while shaking in Innova 4200 incubator, New Brunswick Scientific, 5 ml were put aside for cryopreservation and the rest was used for preparative isolation of plasmid DNA. Unlike ZR Plasmid Miniprep™-

Classic, which is only sufficient for analytical purposes, PureLink™ HiPure Plasmid Filter Midiprep Kit has been used, so the higher amounts of isolated plasmid DNA could be acquired.

It was proceeded as written on the manufacturer's guide, using the "low copy number plasmids" way. The principle is similar to those of mini preparation kit (see 3.1.5). The plasmid DNA was then eluted in the elution tube and it was proceeded for precipitating with isopropanol. It was added 3.5 ml of isopropanol to the eluted plasmid DNA and tube was left for 30 minutes centrifugation at 48000 RPM in Avanti J20 I centrifuge, Beckman. The supernatant was then disposed of and plasmid DNA was resuspended in 70% ethanol. Another 10 minutes of centrifugation at 48000 RPM in the Avanti J20 I centrifuge, Beckman, followed and the supernatant was disposed of as well. The pellet of plasmid DNA stuck to the walls of precipitating tube was then left for approximately 10 minutes, till it was dry and the remaining ethanol evaporated. The clean isolated plasmid DNA was then dissolved in 200 µl of PCR water, thus gaining the first wash. After transferring the first wash into a separate tube, another 100 µl of PCR water was poured into precipitating tube, dissolving the remnants of plasmid DNA to obtain the second wash.

The concentration measuring was also performed, using Colibri, Titertek Berthold. The instrument estimated the concentration of 735.68 ng/µl plasmid DNA in the first wash and of 439.51 ng/µl plasmid DNA in the second wash. Small amount of product (5 µl) was utilized in electrophoresis on 1% agarose gel as depicted in the Fig. 6. The first wash is denoted as number 1, the second wash as number 2.

Figure 6 Gel after midiprep - the hP2Y13 PK2-ARMS2



3.1.6 DNA sequencing

All samples were sent to GATC Biotech that provided the information about the sequence. The 1.Q5 sample was ascertained to carry the wanted $hP2Y_{13}$ sequence, this one being then used for transformation (see Fig. 5).

3.1.7 Cryopreservation of transformed bacterial cultures

For the reason of having permanent stock of transformed bacteria with a specific plasmid, the cryopreservation method was used. The 5 ml of bacterial culture which were put aside when doing preparative isolation of plasmid DNA were used for this purpose. The mixture of 800 µl of bacterial liquid culture and 200 µl of 95% glycerin was prepared in the tubes and stored at -80 °C. If the new culture is wanted to be grown, selection medium (LB medium with

50 μg/ml kanamycin) is inoculated by the tip, which has been steeped in the cryopreserved bacterial culture, gaining the liquid culture ready for further preparations.

3.1.8 Agarose gel electrophoresis

Agarose gel electrophoresis is effective method enabling separation of various sized DNA fragments, as well as their purification.[44] Upon the agarose gelation, a network of bundles is set up, in which the pores occur.[44] The gel is dipped in the buffer which is important for the actual migration of DNA fragments as its pH should leave the phosphate backbone of DNA chain negatively charged. When the electric field is implemented, the DNA fragments carrying negative net charge are pushed through the pores in the gel to the positively charged electrode (anode).[44] The speed of the motion depends on the size of DNA fragments - leaving bigger fragments in comparison to the smaller behind due to more significant interactions with agarose gel network, on agarose concentration - thicker matrix being for DNA fragments harder passable, as well as on further parameters like DNA conformation, applied voltage, type of agarose or buffer characteristics.[44] The staining dye in gel then enables gel visualization after separation under UV light.

For purposes of this work, 1% agarose gels were prepared. Agarose was melted in appropriate volume of 1X TAE buffer, using ordinary microwave. Afterwards, 2.5 μl GelRed™ was added to the melted gel, which was then mixed and poured in the prepared form. The analyzed samples were mixed with 6X DNA Loading buffer in appropriate ratio (1:6) prior to the seeding in the wells. The electrophoresis was then carried out in the span 100-120 V. The addition of GelRed™, which interferes with the DNA fragments, enabled their visualization with UV-light. In order to estimate size of the fragments, the markers were always seeded on gel and run together with analyzed fragments, Lambda marker being suitable for more sizeable fragments whereas Phi marker being intended for smaller ones.

3.2 Cell biological methods

3.2.1 Cell lines passage

In order to preserve optimal conditions for the growing cells, the cells were split every 3-6 days, which depended mostly on the splitting ratio. The lower the ratio, the fewer days till another splitting took its place.

At the beginning, the old growth medium was pipetted out from the flask and disposed of. Having the room temperature, 10 ml of PBS was pipetted in the flask. It was poured on the side of the flask opposite to the cells, in order not to stress them. The cells were then carefully washed by swinging the flask into the sides for a few times. The PBS was then pipetted out from the flask, being then replaced by 3 ml of Trypsin/EDTA-Na_2 (0.05 %/0.6 mM) (TE), which was incubated in the room temperature some time beforehand to warm up a little bit. The cells with TE were put in the incubator for 1-3 minutes, till the cells got detached from the flask walls. The shortest possible exposure of cells to TE was pursued. The cells were shaken down from the walls by reasonable flask walls hitting. The growth medium was then added to the flask in the appropriate volume, remaining cells on the walls were washed down

and this cell suspension was mixed. This addition also effectively ended the effect of trypsin to the cells. In the end, appropriate volumes from this mixture, according to chosen ratio, were transferred to new flasks, already filled with fresh growth medium. These flasks were then incubated in 37 °C, awaiting their further application.

3.2.2 Cryopreservation of cell lines

As the cell lines have only limited efficient working lifespan, it is fairly useful to prepare a stock from the passages. When these cells are needed, there is no necessity to order new cells from distributor or to make again a new transfection to prepare working cells lines. Their cryopreservation is time, money and work-saving.

Before the actual procedure could be accomplished, the cells in big flasks designated for cryopreservation were firstly grown almost confluent (approximately 90 %). On the day of preparation, the old growth medium was disposed of into the waste and its remnants were washed away by the 10 ml PBS. After the PBS was disposed of as well, 3 ml of TE were poured in instead. In a short time of incubation, usually 1-3 minutes, the cells detached the flask walls. The effect of trypsin was ended by addition of 7 ml growth medium. The remaining cells on the walls were washed down and the cell suspension has been mixed. The content of the flask was then transferred into 50 ml Falcon tube and the volume was filled in up to 30 ml. During the follow-up 5 minutes centrifugation at 1200 RPM, the freezing mixture, consisting of 90 % FCS and 10 % DMSO, has been prepared. The DMSO concentration in the final vial volume is essential for cryopreservation as it works as anti-freezing agent. On the other hand, its cytotoxicity compromises use of high concentrations. Its 10% concentration brings in the adequate anti-freezing effect with bearable shortcomings. After the centrifugation has been done, the medium was removed and the pelleted cells were resuspended in the 4 ml of freezing mixture per each flask. 1 ml aliquots were prepared from this cell suspension. The vials were labeled and put into -80 °C freezer.

3.2.3 Revitalization of cell lines

For cell culture preparation from cryopreserved cell lines, the 1ml aliquot was thawed in 37 °C water bath. The cell culture has been transferred to 15 ml Falcon tube and the volume has been adjusted to 10 ml by addition of antibiotic free growth medium. After follow-up 5 minutes 1200 RPM centrifugation, the supernatant containing cytotoxic DMSO was disposed of and the pellet was resuspended in the antibiotic free growth medium and transferred into a big cell culture flask. Antibiotic free growth medium was then substituted by standard selective growth medium with the antibiotics after an overnight incubation (see Tab. 11, 12, 13). It took approximately one week till the cells were ready for further proceeding.

3.2.4 Counting cells with a haemocytometer

To assess the number of the cells for given cell culture methods or experiments, the Neubauer counting cell chamber has been used. It is composed of 2 chambers and each of those is further divided into 9 squares (1x1 mm). The height difference between two external supports

and the central support creates a gap of 0.1 mm between the squares and placed cover glass. Total volume over each square is then 0.1 mm^3 and equals 1 ml. When cell number was needed, each chamber was fed by 10 µl of detached cells (using TE or dissociation buffer). Due to capillary effect, the gap between the cover glass and chamber base filled up completely with the sample. The final number of cells per ml is the mean of counts per square multiplied by 10^4.

3.2.5 Transfection of eukaryotic cells (stable cell lines generation via lipofection)

The transfection is process in which the DNA is introduced into eukaryotic cells. There are basically two types of transfections, the transient and stable transfection. The former method generates transfects only for certain span of time, whereas the latter method integrates the plasmid DNA right into cell genome, thus expressing the introduced gene long-term.

For plasmid DNA carrying human $P2Y_{13}$ gene, the cationic liposome-mediated gene transfer has been used, in order to prepare stable cell-line expressing the wanted $P2Y_{13}$ gene. The plasmid DNA, which physiologically carries net negative charge, is entrapped by cationic liposome.[45] This helps by overcoming the electrostatic repulsion of cell membrane, as nucleic acids and cell membrane are of an even charge.[45] The mixture of plasmid DNA and Lipofectamine 2000, which was used for this lipofection, should be prepared in the time of transfection.

Two transfections were performed in the presented work. Empty Chinese hamster ovary cells (CHO) for beta arrestin assay were cultivated in the F12 medium with addition of FCS (10 %), penicillin and streptomycin (1%) and Hygromycin B (0.9 mg/ml), in order to keep the β-arrestin cassette linked to EA tag (Tab. 10). The cells were seeded in the span of $1\text{-}2x10^6$ cells in the cell culture flasks (25 ml) the day before transfection, so they could grow to at least 90 % confluency. On the next day, the medium was changed for 6.25 ml antibiotic free F12 medium with addition of FCS (10%) only (Tab. 11). Mixtures of Lipofectamine 2000 with pure OptiMem medium were prepared and incubated at room temperature for 5 minutes. Meanwhile, the mixtures of DNA with pure OptiMem medium were prepared in vials as well. The lipofectamine mixtures were then added to vials containing DNA mixture and incubated for the next 20 minutes at room temperature. Finally, the transfection mixtures were added to labeled small flasks. On the next day, the medium has been changed to selection medium containing F12 medium with addition of FCS (10%), penicillin and streptomycin (1%), Hygromycin B (0.9 mg/ml) and G_{418} (800 µg/ml) (Tab. 12). The selection medium was changed every 2 or 3 days, till the stable transfected cell pool was generated. Always using the selection medium, the cells remained under permanent selection pressure.

Transfection of empty CHO β-arrestin cells with $hP2Y_{13}$ PK2-ARMS2 construct (termed as DNA in the Tab. 32) was performed while using various amounts of prepared construct which were added in the transfection mixtures. Numbers of cells that were seeded prior to transfection in the flasks were varied as well. In the first transfection, it was counted with DNA concentration of 0.6 µg/ml, in the second transfection it was counted with concentration of 0.8 µg/ml. The transfection protocols are illustrated in the Tab. 32.

Table 32 Transfection protocols

1st Transfection	
$1x10^6$ cells seeded	37.5 µl Lipofectamine 2000 + 587.5 µl OptiMem
	25 µl DNA (15 µg) + 600 µl OptiMem
$1.5x10^6$ cells seeded	25 µl Lipofectamine 2000 + 600 µl OptiMem
	17 µl DNA (10 µg) + 608 µl OptiMem
2nd Transfection	
$1x10^6$ cells seeded	37.5 µl Lipofectamine 2000 + 587.5 µl OptiMem
	18.8 µl DNA (15 µg) + 606.2 µl OptiMem
$1.5x10^6$ cells seeded	25 µl Lipofectamine 2000 + 600 µl OptiMem
	12.5 µl DNA (10 µg) + 612.5 µl OptiMem
$2x10^6$ cells seeded	25 µl Lipofectamine 2000 + 600 µl OptiMem
	12.5 µl DNA (10 µg) + 612.5 µl OptiMem

The CHO $P2Y_{13}$ PK2-ARMS2 cell lines has been generated and prepared for the testing.

3.2.6 Preparation of monoclonals

Preparation of monoclonals is a method, by which a cell line from one single cell is obtained. The culture is then no more a mixture of various clones. This method can be used for example in selecting the clones which are suitable for the assay.

The flask was disposed of the old medium, its remnants washed away by 10 ml PBS and cells trypsinized by 3 ml TE. After short incubation (1-3 minutes), the volume was filled in to 5 ml with selection medium, mixed and transferred to a Falcon tube (15 ml). Starting with the very preparation, detached cells had to be counted. The Neubauer cell counting chamber has been used. The obtained number was important for the dilutions which were made to get just 100 cells in the seeding mixture. In the first step, 100 µl of counted detached cell culture was added to 9900 µl medium (see Tab. 12). This resulted in 1:100 dilution of the culture (the number of cells per ml was 100 times lower than the number of original cell culture). After precise mixing, 100 µl of this diluted cell culture was added to 9900 µl medium again. Another 1:100 dilution was made (the number of cells per ml was 10000 times lower than the number of original cell culture). The final cell culture for seeding mixture with only tens of cells was prepared after precise mixing.

Around 20 ml of seeding mixture were needed to get 200 µl of this mixture per each well of transparent 96-wells cell culture plate. The seeding mixture was obtained by mixing 20 ml of selection medium (Tab. 12) with the amount of final cell culture corresponding to 100 cells, quite matching with the needed conditions. Steady mixing the reservoir, the seeding mixture was seeded to a transparent 96-wells cell culture plate in two steps (100 µl + 100 µl in each well), so it was perfectly mixed. The plate was incubated at 37 °C till the cultures were grown. This took approximately two weeks.

Ideally, there should be only one cell culture in the well which stems from the just one seeded cell. If it was the case, the content of the well was eligible for further steps. However, there

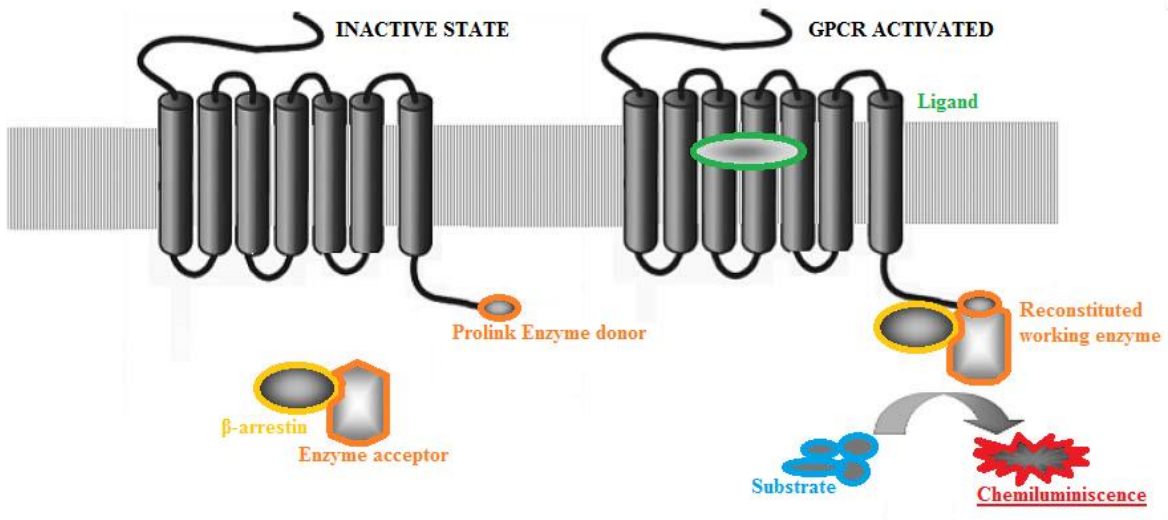
were sometimes no colonies at all or two colonies in the well. In the first case, no cell was seeded or it was unable to survive. In the second case, more than one cell was seeded, thus making the well content ineligible for further proceeding as it is not monoclonal. The wells containing just one colony were circled by marker pen. These were then disposed of old medium washed by 200 µl PBS and incubated for 10 minutes at 37 °C in TE, so the cells detached the walls of the wells. 24-wells cell culture plate was filled by 500 µl of selection medium and the trypsinized cells were transferred to it. After the cells grew confluent, the same procedure has been repeated, transferring cells onto 6-well cell culture plates, filled by 1000 µl of selection medium. After the cells reached confluency, the old medium was removed, the remnants washed by 500 µl PBS followed by 10 minutes incubation with 300 µl TE at 37 °C. Meanwhile, the cell culture flasks (25 cm^2) were filled with 6 ml selection medium, to which the trypsinized cells were transferred. Finally, the test plate was seeded – 6 wells for each monoclonal as well as for wild type which was used as standard. Wild type is term for cells, from which the monoclonals have been originally prepared. The monoclonals, which would prove to have better ratio between agonist supplied cells and the negative control than wild type, would be considered as a positive result.

3.3 Cell-based experiments

3.3.1 Beta arrestin assay

β-arestin assay is a whole cell functional assay, measuring GPCR activity by interaction detection of the β-arrestin with the activated GPCR.[46] In the system presented by DiscoverX PathHunter™, the GPCR is fused on C-terminus with an altered β-galactosidase enzyme fragment (enzyme donor).[46] The cell line is expressing β-arrestin coupled with corresponding β-galactosidase deletion mutant (enzyme acceptor). Upon activation of GPCR with ligand, β-arrestin is recruited and both parts of enzyme reconstitute the original β-galactosidase.[46] The substrate hydrolysis catalyzed by this enzyme generates a chemiluminiscent signal, which can be measured.[46] The principle of the assay is shortly and clearly depicted in the Fig. 7.

Figure 7 Principle of β-arrestin assay (modified from Van der Lee et al. 2008)[46]



3.3.1.1 Agonist screening

Firstly, the cells had to be seeded on 96-well cell culture plate (Nunclon™Delta Surface). For this purpose, the cells had been grown to at least 90-95 % confluency. The original medium was removed and the remnants were washed by 10 ml PBS. As TE would influence the assay results, 10 minutes incubation with 10 ml of dissociation buffer was used for detaching the cells from the flask walls. The detached cells in dissociation buffer were then transferred into Falcon tubes (15 ml) and centrifuged at 1200 RPM for 5 minutes. The supernatant was then carefully removed and the cell pellet was resuspended in 5 ml assay medium (OptiMem, 2% FCS, 1% penicillin/streptomycin, Hygromycin B 300 µg/ml and G_{418} 800 µg/ml) (see Tab. 14). The cells were seeded on 96-well cell culture plate, in the number of 20000-40000 cells per well in the total volume of 90 µl in each well. The seeded plate was then incubated overnight at 37 °C, 5 % CO_2.

Screened compounds were seeded to test plates always in 8 various concentrations (e.g. 10 µM, followed by 3 µM, 1 µM, 0.3 µM and so on) or in 4 various concentrations in pre-screening, either in doublet or triplet on the next (testing) day. However, the tested compounds were always prepared beforehand as 10 mM stock in DMSO and stored in -20 °C. As final concentration of compounds should be in µM and nM values, these were after its thawing diluted in DMSO and pure OptiMem medium in the two separate steps. In the end, the diluted compound in various concentrations was always added in the amount of 10 µl to 90 µl of seeded cells, so that the final volume in every screened well of a tested plate was 100 µl. RA-II-150 was used as a positive control, having 10 µM concentration in the final 100 µl well total volume. The wells containing 90 µl of seeded cells, which were intended to be a negative control, were filled in to well total volume of 100 µl by buffer (DMSO/OptiMem).

The test plates were incubated at 37 °C for 90 minutes after adding the screened compounds. A short time before the end of this period, detection reagent has been prepared. The solution of Tropix® Emerald-II™ (1.1 ml), Tropix® Galacton-Star® Substrate (0.22 ml) and lysis buffer (4.18 ml) was mixed (volumes for completely seeded 96-well cell culture plate). The vial, in which the mixture was made, was protected from light by tinfoil coating as some additives are light-sensible. The light in the laboratory was off as well. Detection reagent enhanced the measured signal and was always added in the amount of 50 µl per well. The light in the laboratory was off during the detection reagent supplementation and after the addition was done, the whole plate was packed in the tinfoil. After 60 minutes incubation at room temperature, the assay measurement was performed on Mithras LB 940, Berthold Technologies. The data processed as excel files were transformed to concentration-response curves using Prism® 5, GraphPad software.

3.3.1.2 Antagonist pre-screening

Similarly to agonist screening, the cells had to be seeded on 96-well cell culture plate (Nunclon™Delta Surface) in the beginning. For this purpose, the cells had been grown at least 90-95 % confluent. The original medium was removed and the remnants were washed by 10 ml PBS. As TE would twist the assay results, 10 minutes incubation with 10 ml of

dissociation buffer was used for detaching the cells from the flask walls instead. The detached cells in dissociation buffer were then transferred into Falcon tubes (15 ml) and centrifuged at 1200 RPM for 5 minutes. The supernatant was then carefully removed and the cell pellet was resuspended in 5 ml assay medium (OptiMem, 2% FCS, 1% Penicillin/Streptomycin, Hygromycin B 300 µg/ml and G_{418} 800 µg/ml) (Tab. 14). The cells were seeded on 96-well cell culture plate, in the number of 20000-40000 cells per well in the total volume of 94 µl in each well. The seeded plate was then incubated overnight at 37 °C, 5 % CO_2.

Screened compounds were diluted on 96-well cell culture plate in DMSO as 1000 µM stock, every compound having exactly one well. The plate was stored in freezer, so it was thawed at 37 °C in the cell plate heater before the use. Meanwhile, the 60 µM RA-II-150 stock solution needed for the assay was prepared from original 10 mM stock. Starting the actual assay, 1 µl antagonist in DMSO was added to seeded cells and the cell plate was then incubated for 1 hour in 37 °C. In the following step, 5 µl of 60 µM RA-II-150 stock were added to wells with tested antagonists and positive control wells, gaining the final 3 µM RA-II-150 concentration in the 100 µl total volume of these wells. 5 µl OptiMem medium were used for negative control wells then. For a better clarity, the preparation protocol is attached in the Tab. 33. Eventually, the cell plate was incubated for other 90 minutes at 37 °C. RA-II-150 was always used as positive control and the buffer (DMSO/OptiMem) as negative control.

Table 33 Preparation protocol for β-arrestin antagonist pre-screening

Antagonist wells	94 µl cells	+ 1 µl antagonist, 60 min, 37 °C + 5 µl RA-II-150 (60 µM stock), 90 min, 37 °C
Positive control wells	94 µl cells	+ 1 µl DMSO, 60 min, 37 °C + 5 µl RA-II-150 (60 µM stock), 90 min, 37 °C
Negative control wells	94 µl cells	+ 1 µl DMSO, 60 min, 37 °C + 5 µl OptiMem medium, 90 min, 37 °C

The detection reagent was prepared shortly before the 90 minutes long incubation ended, using the same constituents (Tropix® Emerald-II™, Tropix® Galacton-Star® Substrate and lysis buffer) as well as the ratio (1.5) like in an agonist assay. The course of steps by the detection reagent addition was same as in the agonist screening (see 3.3.1.1). The data processed as excel files were transformed to % of inhibition (10 µM antagonist compared to 3 µM RA-II-150 positive control) using Prism® 5, GraphPad software.

4 Results

4.1 The hP2Y$_{13}$-PK2 ARMS2 construct transfected to CHO cells in β-arrestin assay

The prepared construct of hP2Y$_{13}$-PK2 ARMS2, which was transfected into empty CHO β-arrestin cell lines, was put through testing with 2-MeS-ADP, a potent agonist of P2Y$_{13}$. The cell lines were screened in triplet at seven progressively decreasing concentrations, starting from 100 µM and ending with 0.1 nM. A signal was generated, its response against negative control (the buffer) was not sufficient though. A ratio between the activities induced by highest concentration of agonist compared to basal activity of negative control was much

lower than needed value of 1.5, compromising use of this cell line for screening other compounds.

4.2 Monoclonals prepared from hP2Y$_{13}$-PK2 ARMS2 expressing CHO cells

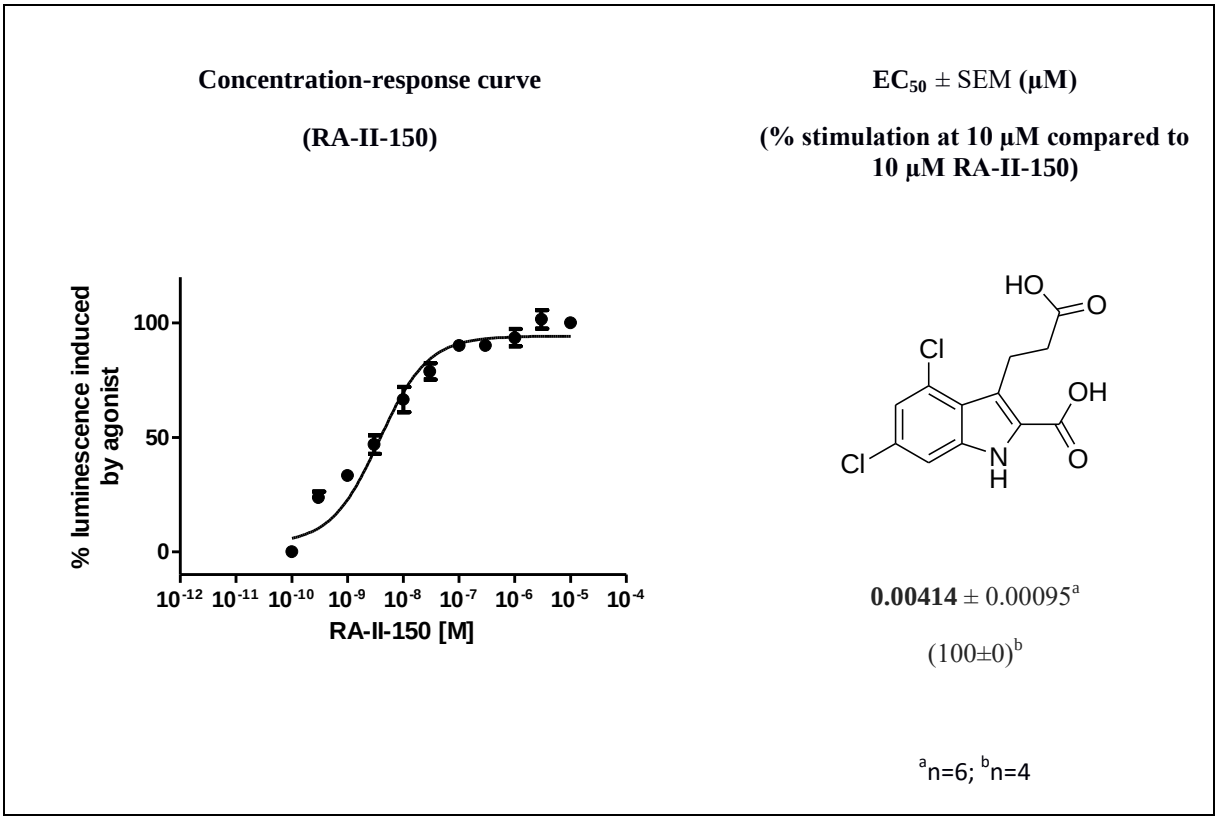
In order to put the β-arrestin assay into operation for hP2Y$_{13}$, the monoclonal cell lines were prepared with an outlook to grow competent clone of cells for the assay which would provide sufficient response and ratio between agonist supplied cells and negative control. At the best, the prepared monoclonal cell lines were only comparable with the wild type, thus bringing in no advantage for compound screening.

4.3 GPR17-PK1 cell line showed sufficient activity for screening assays

The GPR17-PK1 expressing CHO cell line was firstly screened with an already known agonist called RA-II-150, in order to prove that it is viable for other compounds testing.[36,39] For the first screening, 8 different concentrations have been used, starting from 10 μM. Screening at lower concentrations was performed as well, in order to obtain complete concentration-response curve (Fig. 8).

The plates for the assay were seeded according to agonist screening procedure (see 3.3.1.1). EC_{50} value ± SEM were portrayed in μM (n=6). The percentage of agonist induced luminescence was ascertained and is related to 10 μM RA-II-150 which served as positive control (n=4). The raw data ratio between positive control values and negative control background values was sufficient for valid measurement (more than 1.5).

Figure 8 Concentration-response curve of RA-II-150



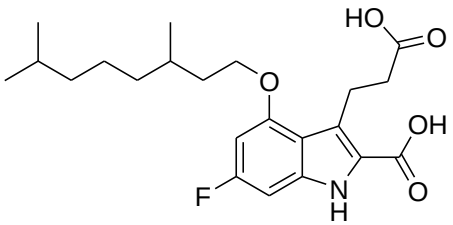
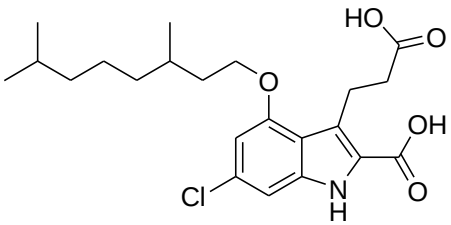
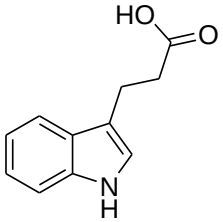
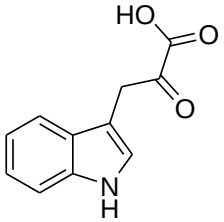
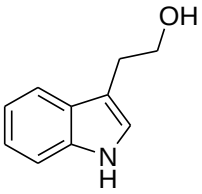
4.4 The GPR17-PK1 is activated by compounds based on RA-II-150 structure

Various compounds sharing certain characteristics with RA-II-150 molecule were screened after the viability of assay for GPR17-PK1 expressing CHO cell line had been confirmed. The compounds were measured in doublets, at least three times each and always in 8 different concentrations. Raw data obtained from cell plate reader in the form of Excel sheet were processed by Prism® 5. The concentration-response curves were constructed and EC_{50} values with percentage of agonist induced luminescence related to 10 μM RA-II-150 were calculated.

Compounds S6067, OLE29, Yazh 183, Yazh 228, Yazh 282, Yazh 336, Yazh 487, Yazh 664, Yazh 665, Yazh 678, Yazh 679, Yazh 690, Yazh 691, Yazh 697, Yazh 698, Yazh 703, Yazh 704, Yazh 706, Yazh 721, Yazh 722 and Yazh 723 were screened in an agonist screening.

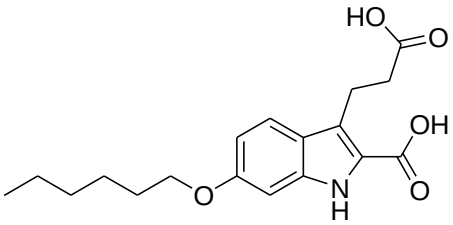
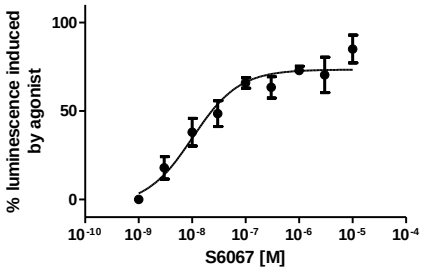
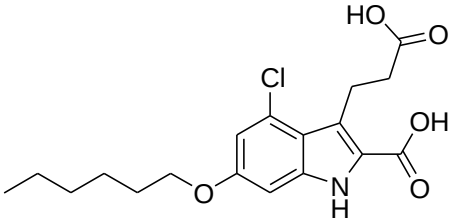
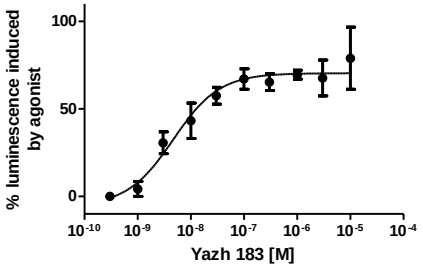
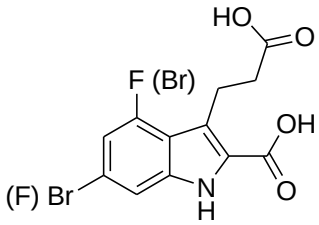
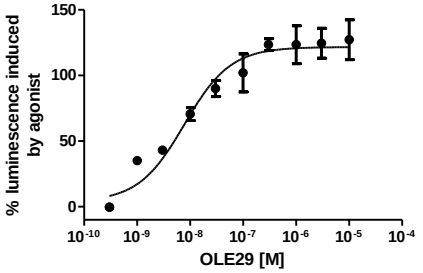
The majority of tested compounds showed substantial activity, although the differences were considerable. Compounds Yazh 664, Yazh 678, Yazh 721, Yazh 722 and Yazh 723 showed only limited activity and the concentration-response curves could not be obtained. The overview of inactive structures is portrayed in the Tab. 34.

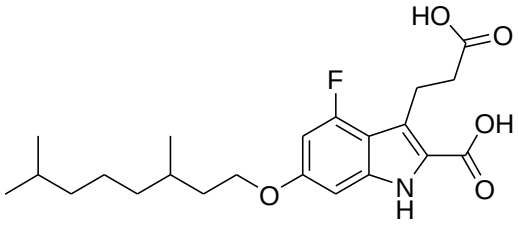
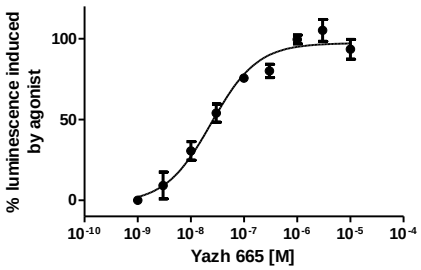
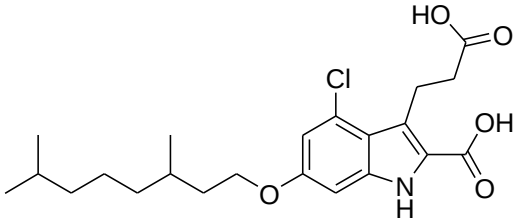
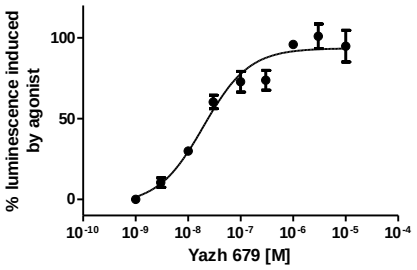
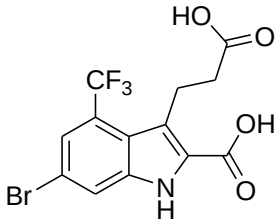
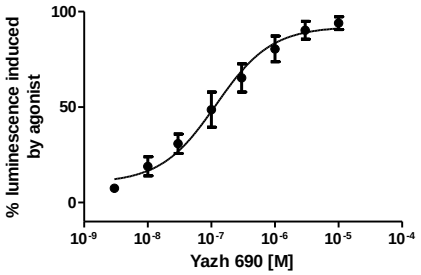
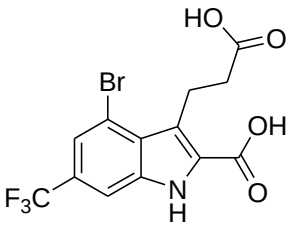
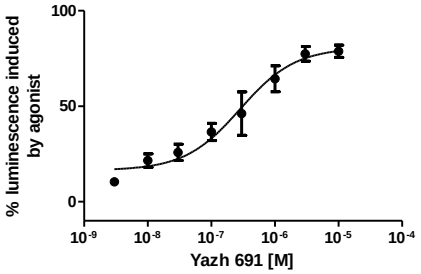
Table 34 The overview of inactive compounds structures.

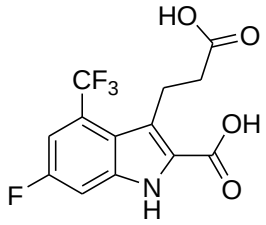
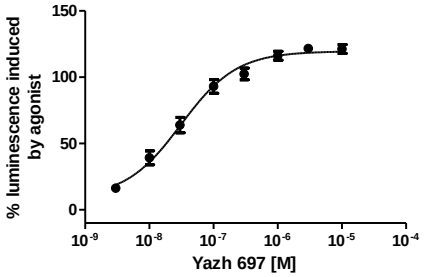
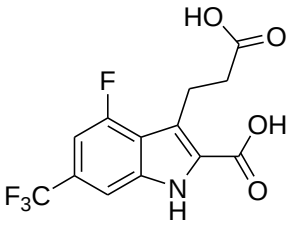
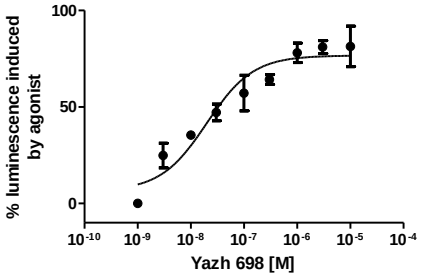
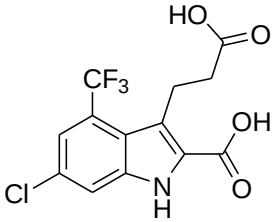
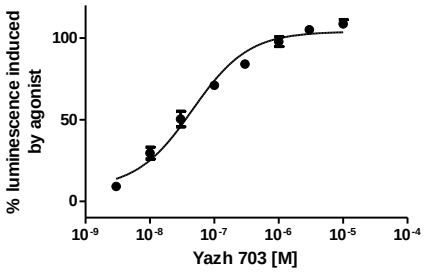
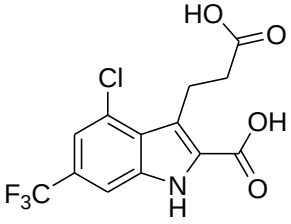
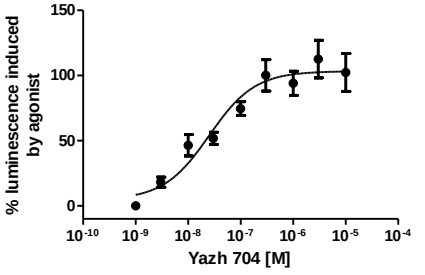
Yazh 664 	**Yazh 678** 
Yazh 721 	**Yazh 722** 
Yazh 723 	

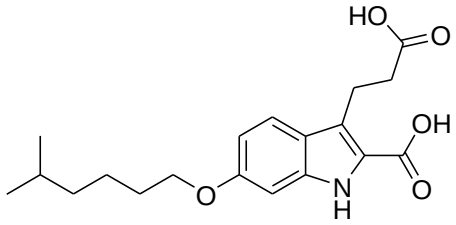
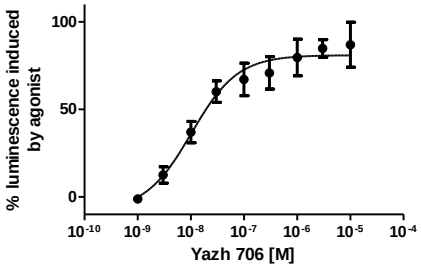
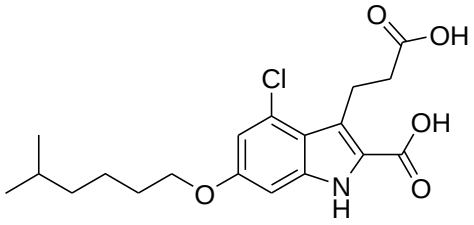
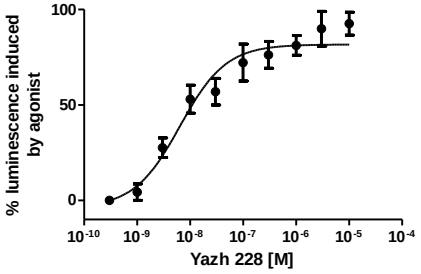
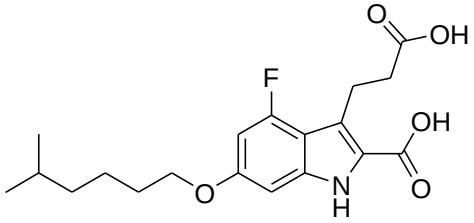
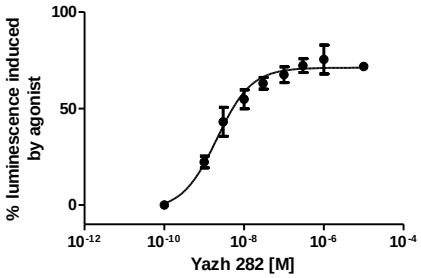
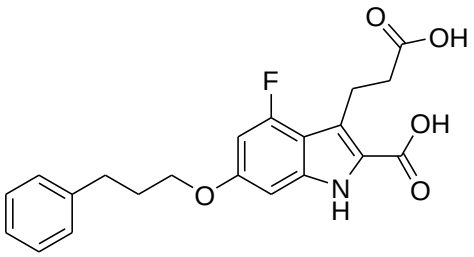
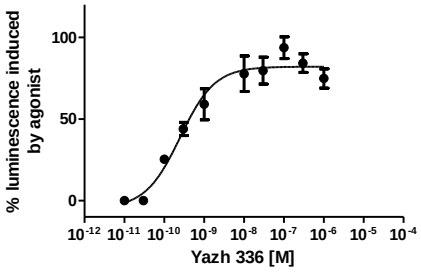
Compounds resembling to RA-II-150 indole-like structure proved to be quite active in the performed agonist screening via β-arrestin assay. The structures of active compounds are presented together with the calculated EC_{50} values and percentages of stimulation to 10 μM RA-II-150 in the Tab. 35. The concentration-response curves are depicted in the Tab. 35 as well.

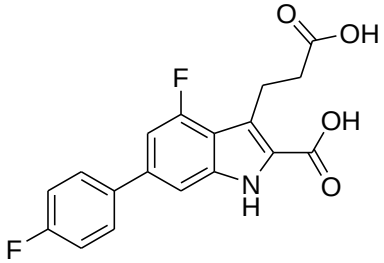
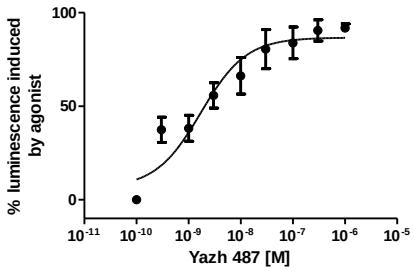
Table 35 List of active compounds on hGPR17

Compound **EC_{50}** ± SEM **(μM)** **(% stimulation at 10 μM compared to 10 μM Ra-II-150),** n=3	**Concentration-response curve** **(% of inducted luminescence related to compound concentration)**
S6067  **0.0102** ± 0.0028 (85±8)	
Yazh 183  **0.00548** ± 0.00154 (79±18)	
OLE29  **0.00618** ± 0.00119 (123±14)[a]	

Compound **EC_{50} ± SEM (µM)** **(% stimulation at 10 µM compared to 10 µM Ra-II-150),** n=3	**Concentration-response curve** **(% of inducted luminescence related to compound concentration)**
Yazh 665  **0.0234** ± 0.0044[b] (93±6)[b]	
Yazh 679  **0.0235** ± 0.0069[b] (95±10)[b]	
Yazh 690  **0.138** ± 0.042 (94±3)	
Yazh 691  **0.391** ± 0.173 (79±3)	

Compound **EC_{50} ± SEM (µM)** **(% stimulation at 10 µM compared to 10 µM Ra-II-150),** n=3	**Concentration-response curve** **(% of inducted luminescence related to compound concentration)**
Yazh 697  **0.0330** ± 0.0085 (121±3)	
Yazh 698  **0.0296** ± 0.0142 (81±11)	
Yazh 703  **0.04501** ± 0.00652 (109±3)	
Yazh 704  **0.0299** ± 0.0102[b,c] (102±14)[b,c]	

Compound **EC_{50}** ± SEM **(µM)** **(% stimulation at 10 µM compared to 10 µM Ra-II-150),** n=3	**Concentration-response curve** **(% of inducted luminescence related to compound concentration)**
Yazh 706  **0.0128** ± 0.0032 (87±13)	
Yazh 228  **0.00878** ± 0.00458[d] (93±6)	
Yazh 282  **0.00250** ± 0.00057[a,b] (75±7)[a,b]	
Yazh 336  **0.000249** ± 0.000031[a] (75±6)[a]	

Compound **EC_{50} ± SEM (µM)** **(% stimulation at 10 µM compared to 10 µM Ra-II-150),** n=3	**Concentration-response curve** **(% of inducted luminescence related to compound concentration)**
Yazh 487  **0.00211** ± 0.00063[a] (92±2)[a]	

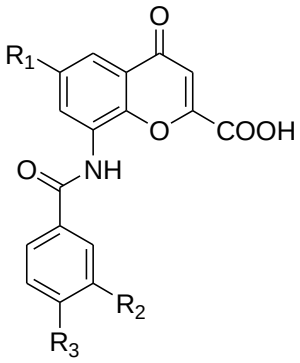
[a] at 1µM;[b] n=4;[c] Yazh 704 is a mixture with its isomer Yazh 703 in the ratio of 10:1;[d] n=5

4.5 The antagonist pre-screening of benzopyran-based compounds on GPR17

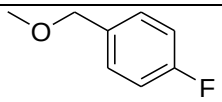
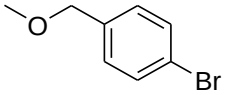
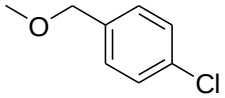
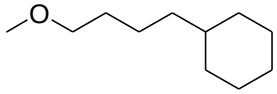
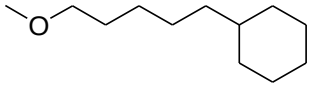
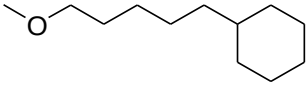
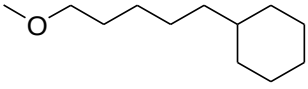
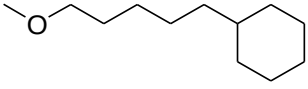
The compounds whose structures are based on benzopyran (4*H*-chromene) were tested in an antagonist pre-screening on GPR17-PK1 expressing CHO cell line in order to obtain information whether these compounds evince antagonist-like behavior (see 3.3.1.2). All the compounds were measured three times and the raw data obtained from the cell plate reader in the form of Excel sheets were processed by Prism® 5. The percentage of inhibition was calculated as a mean at 10 µM antagonist in comparison to 3 µM RA-II-150. If any of the compounds seemed to be a potent antagonist, it would be acceded to a more detailed screening with a full concentration scale in order to obtain full concentration-response curve.

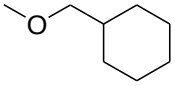
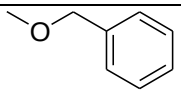
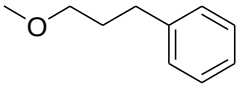
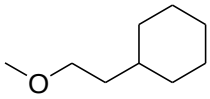
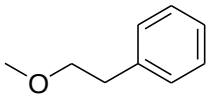
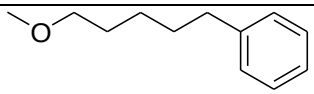
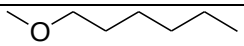
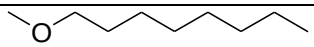
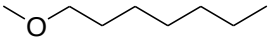
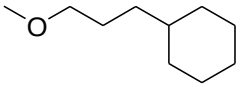
Compounds ANM06, ANM07, ANM20, ANM21, ANM22, ANM23, ANM27, ANM34, ANM40, ANM47, ANM68, ANM77, ANM78, ANM111, ANM158, ANM182, ANM183, ANM250, ANM251, ANM252, ANM263, ANM264, ANM265, ANM267, ANM270, ANM279, ANM280, ANM287, ANM295, ANM296, ANM297, ANM298, ANM299, ANM300, ANM334, ANM335, ANM337 and ANM346 were pre-screened, but no compound proved to be a potent antagonist (data not shown). Thus no further and more detailed testing was not performed. Structures of screened compounds are represented in the Tab. 36, Tab. 37 and Tab. 38.

Table 36 Compounds tested in antagonist pre-screening (A)



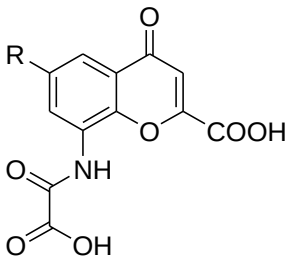
The basic structure (A) of ANM compounds derived from benzopyran (4*H*-chromene)

Compound	R_1	R_2	R_3
ANM20	-Br		-
ANM21	-Br		-
ANM22	-Br		-
ANM27	-Br	-F	$-OCH_3$
ANM34	$-CH_3$	-	$-OCH_3$
ANM40	$-OCH_3$	-	$-OCH_3$
ANM68	$-OCF_3$	-	$-OCH_3$
ANM77	-Phe[a]	-	$-OCH_3$
ANM78[b]	-Br	-	$-OCH_3$
ANM111[c]	$-OCH_3$	-	
ANM158	-	-	
ANM250	-		-
ANM251	-Cl		-
ANM252	-F		-

Compound	R_1	R_2	R_3
ANM263	-		-
ANM264	-		-
ANM265	-		-
ANM267	-		-
ANM270	-		-
ANM279[d]	-Br	-	$-OCH_3$
ANM280	-		-
ANM334	-Cl		-
ANM335	-Cl		-
ANM337	-Cl		-
ANM346	-F		-

[a]Phe=Phenyl; [b]The amide nitrogen is methylated; [c]Ethylester on the carboxylic function in the position 2 of basic 4*H*-chromene structure; [d]Thioketone in the position 4 of basic 4*H*-chromene structure

Table 37 Compounds tested in antagonist pre-screening (B)

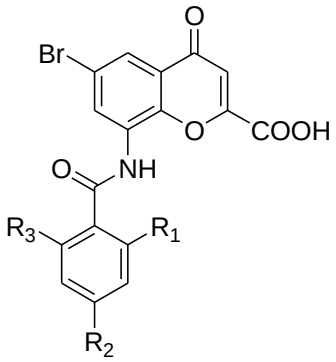


The basic structure (B)

Compound	R
ANM183	-Br
ANM287	-Phe[a]
ANM295	-F
ANM296	-Cl
ANM297	$-OCH_3$
ANM298	$-CH_3$
ANM299	$-CH_2CH_3$
ANM300[b]	-Br

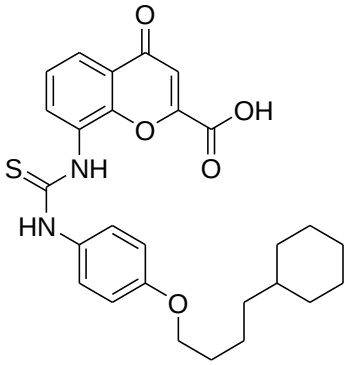
[a]Phe=Phenyl; [b]Thioketone in the position 4 of basic 4*H*-chromene structure

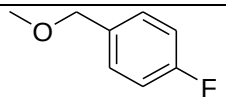
Table 38 Compounds tested in antagonist pre-screening (C)



The basic structure (C)

ANM182



Compound	R_1	R_2	R_3
ANM06	-F	$-OCH_3$	-
ANM07	-F	$-OCH_3$	-F
ANM23		-	-
ANM47	$-OCH_3$	$-OCH_3$	-

5 Discussion

As far as human $P2Y_{13}$ receptor is concerned, the established recombinant CHO cell lines expressing the receptor did not work as expected. Though certain response was obtained in the performed β-arrestin assay, the observed results did not support further step - compound screening, as the ratios between cells activated by 100 μM 2-MeS-ADP and negative control was way too low to conduct any measurements. As the follow-up monoclonal cell lines preparation did not yield any better result, the question arises if actually the $P2Y_{13}$ was expressed by the prepared CHO cell lines. The methods for protein assessment like Western blotting, ELISA and other could shed more light on the issue, but were not performed because of the time shortage. However, the β-arrestin assay utilization for the noted receptor was not proved false, so that the goal of β-arrestin assay establishment, in which human $P2Y_{13}$ receptor could be more specified by screening its known as well as possible ligands ended up in an inconclusive result.

With regards to human GPR17, the specific patterns between structure and activity were observed, RA-II-150 (EC_{50}=0.00414±0.00095 μM) being the lead compound and model, to which the measurements could be compared (Fig. 8). The compounds Yazh 721, Yazh 722 and Yazh 723 are modifications of the lead structure, which showed only a limited activity and whose concentration-response curves could not be obtained. All these molecules differ from each other only in the side chain region, but foremost one fact is to be taken into consideration - they all lack any substitution on the position 4 and 6 of the basic indole

structure (see Tab. 34). The data obtained from measurements could indicate that substitutions on these positions are essential for GPR17-agonizing compounds. However, the complete substitution on both positions must not unconditionally result in a GPR17 agonist.

The compounds Yazh 664 and Yazh 678 are carrying bulky alkyloxy substituent on the position 4, the position 6 being filled with fluorine or chlorine respectively (Tab. 34). Limited activity of these compounds denotes the fact that nature of the substituents on both mentioned positions is important as well. Most likely, the bulky substituents on the position 4 are not tolerated well by the GPR17 receptor. The only up to date measurements of GPR17 activities on indole-like substances was done by Baqi et al., 2014 and shows that compounds substituted on the position 4 evince somehow weaker agonist behavior if the substituent reaches certain size (dichloro, dibromo, diiodo derivatives in the cited article).[47] They proposed that not only sterical effects, but also electronegativity as well as electronic effects of the substituents play an important role (di(trifluoromethyl), dimethoxy derivatives in the cited article).[47] The activity of dimethoxy derivative measured by Baqi group is interesting when it comes to comparison with previously reported compounds Yazh 664 and Yazh 678 in the presented work as all these coumpounds lack substantial activity, the results obtained in this work being in line with Baqi group results.

Further category of tested compounds consisting of substances carrying halogens or trifluormethyl group on the positions 4 and 6 indicates that the bulkier the substituent on the position 6, the better agonist-like properties of the compound. Compound Yazh 690 with trifluormethyl group and bromine, the former on the position 4, the latter on the position 6 proved to be more potent than its counterpart Yazh 691, which is substituted exactly in the opposite way (EC_{50}=0.138±0.042 μM and 0.391±0.173 μM respectively) (Tab. 35). Bromine is of bigger size than trifluormethyl group and this outcome is in line with experiments performed by Baqi group, where the bromine and iodine lead compound derivatives were tested that were substituted only on the position 6. The compound carrying the bulkier iodine atom was clearly superior to the compound having bromine incorporated on this particular position.[47] Similar situation was observed in the case of the compounds Yazh 697 (EC_{50}=0.0330±0.0085 μM) and Yazh 698 (EC_{50}=0.0296±0.0142 μM). Though not markedly, the second mentioned compound with larger trifluormethyl substituent on the position 6 is more potent than the former one, being substituted by fluorine in this position (Tab. 35). Moreover, the compound Yazh 704 (EC_{50}=0.0299±0.0102 μM) with chlorine on position 4 and bigger trifluormethyl on the position 6 showed to be stronger agonist than the Yazh 703 (EC_{50}=0.04501±0.00652 μM) carrying its substituents in an inverse manner (noted in the Tab. 35).

The alkyloxy substitution of the position 6, where substituent possesses longer lipofilic chain, proved to be an icredibly advantageous modification of the lead compound structure. Whereas the compounds Yazh 664 and Yazh 678, carrying these large lipofilic substituents on the position 4 showed only limited activity, the compounds having them in the position 6 evinced in general strong agonist-like behavior (Tab. 34 and 35 respectively). The compound S6067 (EC_{50}=0.0102±0.0028 μM) is substituted with a long alkyloxy substituent only in the position 6, leaving the position 4 unoccupied. Though weaker than the lead structure RA-II-

150, it seemed to be fairly strong agonist. However, the compound Yazh 183 (EC_{50}=0.00548±0.00154 μM), having the same substituent on the position 6 as S6067, in addition to that being substituted by chlorine atom in the position 4 proved to be comparable to the lead structure RA-II-150 (EC_{50}=0.00414±0.00095 μM) (Tab.35).

The next subgroup of compounds having also this longer alkyloxy substituent on the position 6, but with a branched end in this case, showed similar results. The compound Yazh 706 (EC_{50}=0.0128±0.0032 μM) possesses no substituent on the position 4, the compound S6067 in the previous case alike (Tab. 35). Its counterparts Yazh 228 (EC_{50}=0.00878±0.00458 μM) which has this position occupied with a chlorine atom and Yazh 282 (EC_{50}=0.00250±0.00057 μM) having this position filled with fluorine proved to be stronger than the previously mentioned compound Yazh 706 (Tab. 35). Like in the previous paragraph, the compounds substituted also on the position 4 proved to be stronger. This fact indicates that this position could also play an important role.

The data obtained from measurements indicates that excessive branching of the aforesaid bigger alkyloxy substituent on the particular position 6 has also an effect on the compound activity regarding the GPR17. The compound Yazh 679 (EC_{50}=0.0235±0.0069 μM) and Yazh 665 (EC_{50}=0.0234±0.0044 μM), though being substituted with halogens on the position 4, did not reach as high activities as their corresponding compounds Yazh 228 and Yazh 282 mentioned in the previous paragraph (Tab. 35).

On the other hand, the two compounds, Yazh 336 (EC_{50}=0.000249±0.000031 μM) and Yazh 487 (EC_{50}=0.00211±0.00063 μM) evinced the strongest agonist-like properties, Yazh 336 being the strongest measured compound in the presented work. These two compounds share certain similarity - for example both possess fluorine atom on the position 4 and an aromatic circle in the side chain bound to the position 6 (Tab. 35). This modification of the lead structure, in which an aromatic circle is implemented in the side chain bound to the position 6, seems to be very promising for further GPR17 agonist development. Given that only two compounds in the presented work incorporate such feature in their structure, there is no possibility to assess further relations between structure and activity. More resembling compounds should be tested to uncover other connections.

The wide scale of compounds was tested in the antagonist pre-screening. Their structure showed only a little similarity to already known or putative GPR17 antagonists. Structure of pranlukast, the $CysLTR_1$ antagonist, which is also thought to be a GPR17 antagonist, possesses the closest similarity to the measured compounds, having the same 4*H*-chromene core.[28,36,39] However, the differences between pranlukast and the tested structures are still substantial. For example, pranlukast has tetrazol circle substitution in the position 2 of basic 4*H*-chromene structure, whereas tested compounds possess a carboxyl functions in this position. The substituents on the position 8 of the fundamental 4*H* chromene core differ as well. Despite such a number of compounds tested in the pre-screening, the inhibition induced by these compounds in the pre-screening assays was not high enough for a reasonable full-scale concentration screening of the compounds denoted in the Tab. 36, 37 and 38. This fact

did not allow us to get more information about structure-activity relationship estimation, which would be needed for the better comprehension of GPR17 pharmacology.

An interesting finding acquired from the performed measurements is also the fact that the EC_{50} values obtained via β-arrestin assay were really diminutive in comparison to Baqi group, which used 1321N1-astrocytoma cells recombinantly expressing human GPR17 receptor and calcium mobilization assay in their experiments. Clarification of the phenomenon was not disentangled yet. However, the results obtained in the presented work generally support observations made by Baqi group. The aim of the presented work has been partially fulfilled as the β-arrestin assay enabled closer characterization of chosen compounds acting on the GPR17 receptor. The EC_{50} values were obtained and concentration-response curves were constructed.

It is necessary to remark the fact that the measured data were obtained using only one cell line and one particular screening assay though. Regrettably, experiments on the different cell lines were not performed. Nevertheless, further complex screening of the measured compounds using different approaches would certainly reveal more knowledge about the GPR17, bring in by far more significant insight into GPR17 pharmacology and increase the relevancy of the up-to-date acquired data.

6 Conclusion

The GPR17-PK1 expressing cell line proved to be eligible for β-arrestin assay by running tests with an already known agonist. Novel compounds acting on GPR17 were then found and their activity was measured, successfully using the previously mentioned β-arrestin assay. The obtained data enabled not only getting basic information about potencies of measured compounds, but allowed to a limited extent gain of some insight into the receptor pharmacology via structure-activity relationship. The role of various substituents and their positions in the molecules derived from the lead compound has been evaluated and compared with data in the literature. Despite the fact that number of compounds was screened, the current knowledge of structure-activity relationships remains still bounded and much from it waits for its revelation. It is also necessary to remark that only one screening assay was used. Thus it would be interesting as well as of high importance to see results of the compounds measurements obtained in different assay systems. Nevertheless, the results from the presented work are indicating directions, to which the further exploration could be going.

The analogous cell line for compound screening was prepared for $P2Y_{13}$. Tests were made with an already known agonist like in the previous case. Using β-arrestin assay, the signal was generated and detected. This points out that the method alone was working. However, the generated signal was not sufficient enough to undertake compound screening. Trying to obtain better conditions, the monoclonal cell lines were prepared, yielding regrettably no profit. In the summary, it is not possible to evaluate, whether the method is - or is not - suitable for closer characterization of $P2Y_{13}$ and for determining of agonists or antagonists potencies.

As both denoted receptors bear a certain share on various patophysiological processes, it is likely that more research in this field will be done and will be heard of. The same stands for the mentioned β-arrestin assay. Its elegant principle as well as modest requirements makes from it a valuable tool in pharmacological testing, so the method will most probably linger in the wide spectrum of currently applied assays.

7 Abbreviations

GPCR=G protein coupled receptor, 7-TM=7 trans-membrane region, ECL=extracellular loop, ICL=intracellular loop, GDP=guanosine diphosphate, GTP=guanosine triphosphate, AC=adenylatecyclase, cAMP=cyclic adenosine monophosphate, PKA=Protein kinase A, PLC-β=phospholipase C, PKC=protein kinase C, PCL-β, IP_3=inositol triphosphate, DAG=diacylglycerol, PI3K=phosphoinositide 3 kinase, MAPK=mitogen-activated protein kinases, PAM/NAM=positive/negative allosteric modulator, NAL=neutral allosteric ligand, GRK=G protein coupled receptor kinase, AP2=adapter protein 2, ERK=extracellular signal-regulated kinase, JNK=c-Jun N-termine kinase, LGIC=ligand-gated ion channel, CREB=cAMP response element-binding protein, 2-MeS-ADP=2-methylthioadenosine diphosphate, CysLTR=cysteinyl leukotriene receptor, UDP=uridine diphosphate, MS=multiple sclerosis, Df=*Dermatophagoides farinae*, CHO=Chinese hamster ovary

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