CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

&

UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Chemistry

CHARACTERIZATION OF LIGAND BINDING TO M_1 MUSCARINIC ACETYLCHOLINE RECEPTOR USING FLUORESCENCE ANISOTROPY METHOD

Diploma thesis

Supervisors: Prof. Ago Rinken, PhD.
MSc. Tõnis Laasfeld
PharmDr. Ivan Vokřál, PhD.

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Hana Danková

Declaration

I hereby declare that I am the sole author of this master thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

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Prohlášení

Prohlašuji, že tato práce je mým původním autorských dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci jsou řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu.

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V Hradci Králové, dne Podpis: ……....

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ABSTRAKT

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Studentka: Hana Danková

Školitelé: Prof. Ago Rinken, PhD.

MSc. Tõnis Laasfeld

PharmDr. Ivan Vokřál, PhD.

Téma diplomové práce: Charakterizace vázání ligandů na M_1 muskarinový receptor s použitím metody fluorescenční anisotropie

Muskarinové acetylcholinové receptory (mAChR), které jsou členy superrodiny receptorů spřažených s G-proteiny, regulují životně důležité fyziologické procesy a jsou významnými cílovými receptory ve výzkumu léčiv. Identifikováno bylo pět subtypů těchto receptorů (M_1 – M_5). M_1 mAChR jsou lokalizovány převážně v centrální nervové soustavě a jsou spojovány s patofyziologií neurodegenerativních onemocnění. V posledních letech byly metody s použitím fluorescence často využívané ve studiích ligandů vážících se na receptory. Fluorescenční anisotropie (FA) je homogenní metoda, která je používána na charakterizaci vázání ligandů na receptory.

V této práci jsme vyhodnocovali FA metodu s použitím fluorescenčního ligandu MK342 vázajícího se na M_1 mAChR exprimovaný na bakulovirusových částicích. Fluorescenční ligand se vázal s vysokou afinitou (4,4 nM) na M_1 receptory exprimované v bakulovirusovém vzorku. Screenovány byly afinity (pK_i) jedenácti ortosterických a tří bitopických ligandů, které byly porovnány s dříve publikovanou literaturou. Ve většině případů byly hodnoty afinity zjištěné FA metodou nižší než dříve publikované, ale lineární korelace s $R^2 = 0,95$ dokazuje, že FA metodu je možné použít ve studiích charakterizace vázání ligandů. On-line monitoring kompetitivního vázání bitopických ligandů ukazuje komplexnější způsob vázání, a proto se jeví, že MK342 je možné použít taky ve studiích alosterické modulace.

ABSTRACT

Charles University

Faculty of Pharmacy in Hradec Králové

Department of pharmacology and toxicology

Student: Hana Danková

Supervisors: Prof. Ago Rinken, PhD.

MSc. Tõnis Laasfeld

PharmDr. Ivan Vokřál, PhD.

Title of diploma thesis: Characetrization of ligand binding to M_1 muscarinic receptor using fluorescence anisotropy method

Muscarinic acetylcholine receptors (mAChRs), members of the superfamily of G-protein coupled receptors (GPCRs), regulate vital physiological processes and are important targets in drug research. Five different subtypes (M_1 – M_5) have been identified. M_1 mAChR is mainly distributed in the central nervous system and is linked to pathophysiology of neurodegenerative diseases. In recent years, fluorescent methods have been frequently used in studies of ligand binding to receptors. The fluorescence anisotropy (FA) is a homogenous assay to characterize ligand binding to receptors.

In this work, we have evaluated the FA method with fluorescent ligand MK342 binding to M_1 mAChRs expressed on budded baculovirus (BBV) particles. The fluorescence ligand was binding with the high affinity (4,4 nM) to M_1 receptor in constructed BBV preparation. The apparent binding affinities (pK_i) of eleven classical and three bitopic muscarinic ligands were screened and compared to previously published literature. In most cases, the affinity values of competitors determined in FA assay were lower than previously published but the linear correlation analysis with $R^2 = 0,95$ shows the FA method can be used to characterise ligand binding. The on-line monitoring of competitive binding with bitopic ligands indicates a more complex mode of binding therefore it appears that MK342 can be also used in studies of allosteric modulation.

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1. List of abbreviations

4-DAMP – 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide

AD – Alzheimer's disease

BBV – budded baculovirus

BV – baculovirus

CNS – central nervous system

DAG – diacylglycerol

FA – fluorescence anisotropy

FL – fluorescent ligand

FRET – Förster/fluorescence resonance energy transfer

GPCR – G-protein coupled receptor

GTP – guanosine triphosphate

IC_{50} – half maximal inhibitory concentration, here concentration of inhibitor, which displaces 50% of the specific binding of fluorescently labelled ligand

IP_3 – inositoltrisphosphate

ivp – infectious viral particle

K_d – equilibrium dissociation constant

LAMA – long acting muscarinic antagonist

$M_{1\text{-}5}$ – muscarinic acetylcholine receptor subtypes 1-5

mAChR – muscarinic acetylcholine receptor

McN-A-343 – 4-(m-chlorophenyl-carbamoyloxy)-2-butynyltri-methylammonium

MOI – multiciplity of infection

Na-HEPES – buffering agent, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate

NAM – negative allosteric modulator

NMS – N-methylscopolamine

ODV – occlusion-derived virus

PAM – positive allosteric modulator

SAMA – slow acting muscarinic antagonist

Sf9 – insect cell line derived from species *Spodoptera frugiperda*

2. Introduction

Muscarinic acetylcholine receptors (mAchRs) are a group of membrane proteins that belong to a superfamily of G-protein coupled receptors (GPCRs). The function of mAChRs is regulated by endogenous neurotransmitter acetylcholine. Five subtypes ($M_1 – M_5$) have been identified. Wide distribution of these receptors in cardiovascular system, gastrointestinal system, respiratory system, central nervous system and many other tissues demonstrates their indispensable role in maintaining homeostasis in the human body. Of these five subtypes, mainly M_1 receptor is located in many brain regions and is important for cognitive processes. Its impairment is often linked to pathophysiology of Alzheimer's disease and schizophrenia. In recent years, emerging number of promising drugs in treatment of cognitive deterioration have been tested both on animal models and in clinical trials. However, most of them failed to proceed to clinical practice because of ineffectiveness or due to undesirable adverse effect resulting from non-selectively targeting peripheral muscarinic receptors (Wess *et al.*, 2007, Felder *et al.*, 2018).

In the beginning of the drug development, suitable ligands must be well characterized in binding studies in terms of their kinetic properties and modes of binding to the target receptors. Radioligand binding assay is a predominant method to study the ligand- receptor interactions to date, despite having numerous disadvantages. Lately, various alternatives to radioligand binding assay were developed, resolving some of these limitations. One of such are fluorescence-based methods, including fluorescence anisotropy (FA) assay. FA method bases on phenomenon that fluorophore emits polarized light, if it has been excited with plane-polarized light. The polarization of the emitted light depends on the fluorescently labelled ligand's rotational freedom and its fluorescence lifetime. When the tracer is free, it rotates in the solution within fluorescence lifetime and causes depolarization of formerly polarized light, resulting in low anisotropy signal. On the other hand, tracer rigidly bound to the receptor changes the degree of polarized light to lesser extent, which results in high anisotropy signal. The FA assay is therefore ratiometric, as both concentration of the free tracer and concentration of the receptor-bound tracer contribute to the anisotropy signal. The course of the binding can be observed directly on- line with no further separation steps required. (Rinken *et al.*, 2018).

Ligand binding studies are performed on suitable overexpression system with high signal-to-noise ratio. Baculovirus expression system is a suitable tool to overcome problems with mixture homogeneity mixture and stability over time, that is common in studies with whole cells or membrane preparations. Uniform budded baculovirus (BBV) particles carry the membrane of the host cell with expressed proteins of interest, which are correctly oriented in the sample mixture. Additionally, BBVs can be produced on large scale and since they are not infectious to humans, they can be handled in biosafety level 1 conditions (Veiksina *et al.*, 2014).

In optimized assay format, FA method can be applicable in high-throughput screening of novel drugs. The crucial step is to find a highly affine and selective tracer with suitable kinetic properties towards studied receptor. Number of well-characterized fluorescent ligands for some target proteins including GPCRs are already commercially available (Iliopoulos- Tsoutsouvas *et al.*, 2018). In this thesis, we studied binding of fluorescent ligand MK342, a TAMRA-labelled dibenzodiazepinone derivative selective to muscarinic receptors.

3. Theoretical part

3.1. G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a superfamily of membrane proteins, the largest, most diverse and highly druggable group of proteins. They have an indisputable role in human physiology and pathophysiology of many diseases. To date, more than 800 members have been identified, some of which are orphan receptors i.e. with unknown endogenous ligand. There are five major families of GPCRs, the Rhodopsin-like family (also known as class A) being the largest and the most scrutinised one. They all consist of 7- transmembrane domains connected by extracellular and intracellular loops. The spectrum of ligands, that target GPCRs is extremely diverse: from photons and ions to lipids and proteins. The first published three-dimensional model of class-A GPCR bovine rhodopsin resolved the alignment of transmembrane helices and served as the exemplar model to derive structure of other receptors of this class. Rhodopsin-like family possess a short N-terminal tail located extracellularly. The binding pocket is located in the transmembrane domain, available to the ligand reaching from the extracellular site (Costanzi *et al*., 2009, Fronik *et al.*, 2017).

The process of signal transduction is initiated by ligand binding to receptor and subsequently rearrangement of receptor transmembrane helices, so they can interact with the intracellular G-protein. G-proteins are heterotrimeric protein complexes consisting of α, β and γ subunits. Upon activation, the α- subunit of G-protein release guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP), which leads to dissociation of α-subunit from dimeric βγ complex. α- subunit or βγ complex then interacts either with ion channels or membrane-bound enzymes, mainly adenylyl cyclase or phospholipase C (Table 1). Opening of ion channel leads to influx or efflux of ions, usually where rapid action is needed (e.g. potassium channels in heart muscle). Activation of enzyme-linked G-proteins leads to more complex processes with involvement of second messengers (cyclic adenosine monophosphate (cAMP) or inositoltrisphosphate (IP_3) and diacylglycerol (DAG)), that can lead to modulation of many processes including gene expression. As the α-subunit itself has intrinsic GTPase activity, it can inactivate itself by hydrolysis of GTP to GDP and unbind from the effector protein. Signal transmission is therefore terminated by reconnection of α- subunit to βγ complex (Alberts, 2005).

Table 1 G-protein subtypes and signal cascade

Gα-protein subtype	Effector	2nd messenger/action
G_q	Phospholipase C	Inositol trisphosphate, diacylglycerol, Ca^{2+} release from endoplasmatic reticulum
G_s	Adenylyl cyclase activation	cAMP production
G_i	Adenylyl cyclase inhibition	Decrease in cAMP production
G_o	Effectors mainly interact with βγ complex	Ion channels regulation

Source: Rang & Dale, Pharmacology, 8th edition (2016)

Since the GPCR class-A is evolutionarily very old, the orthosteric binding site is highly conserved among different subtypes. Common feature of the class-A are 7- transmembrane helices with orthosteric binding pocket located between these helices. Many structures indicate the presence of allosteric binding site located remotely from the orthosteric binding site. It was proposed some receptors form oligomers. However, there are some differences in the structure, mainly in the 2nd extracellular loop. As a consequence, drugs often lack the selectivity for one receptor subtype and bind to other types as well. Current trend is the development of bitopic/dualsteric ligands, where both orthosteric and allosteric site are occupied by the ligand forming a ternary complex, resulting in higher affinity and subtype selectivity (Kratochwil *et al.*, 2011).

To date, 306 high-resolution crystal structures of this class of receptors have been identified. strongly contributed to the novel drug design and basic science. The crystal structures have helped to extract the exact structural elements (ligand-contacting residues, conserved motifs) and have elucidated detailed molecular mechanism of ligand binding to receptor in the active state (bound to agonist), inactive state (bound to antagonist) and allosteric modulation. Computational methods, especially molecular dynamic simulations

or virtual ligand screening have driven the progress even further with less experimental requirements (Pándy-Szekeres *et al.*, Shonberg *et al.*, 2014).

By 2017, 34% of all FDA-approved pharmaceuticals and more than 321 drugs tested in clinical trials target this class of receptors. The spectre of diseases is extremely variable and diseases of the central nervous system, diabetes and obesity are the most frequent indications of drugs, that target GPCRs (Hauser *et al*, 2017).

3.2. Muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors (mAchRs) belong to the Rhodopsin family of GPCRs. Neurotransmitter acetylcholine is the endogenous ligand binding to the receptors' orthosteric binding site. Five mammalian subtypes of muscarinic receptors (M_1- M_5) have been identified. While M_1, M_3 and M_5 couple to the G_q-protein subtype leading to activation of phospholipase C or D and increase in cytosolic Ca^{2+} concentration, M_2 and M_4 subtypes prefer coupling with G_i or G_o-protein subtypes, resulting in the inhibition of adenylyl cyclase. Receptors are expressed in both the central and the peripheral nervous system (vital part of the parasympathetic innervation), as well as in the peripheral tissues – the effectors of parasympaticus. Usually more than one receptor subtype is expressed in a single tissue, characteristically one predominating over the other, indicating its typical physiological response (Rang *et. al.,* 2016) (Table 2).

In the central nervous system (CNS), processes connected with cognition, sensory feeling, motor activity and behaviour are regulated mainly by M_1, M_4 and M_5 subtypes. M_2 and M_3 are located both centrally and peripherally, mainly known for their regulation of heart functions, gastrointestinal tract and glandular secretion (Table 2). The impairment of these receptors and/or cholinergic transmission is the underlying cause of broad spectrum of diseases, including Alzheimer's disease, schizophrenia, asthma and chronic obstructive pulmonary disease among many others (Scarr, 2012; Coulson & Fryer, 2003).

Table 2 Location and function of muscarinic receptor subtypes

Receptor subtype	Predominant location	Physiological response
M_1	Cerebral cortex, hippocampus, glands	Cognition enhancement, gastric secretion
M_2	Heart atria, CNS	Decrease in heart rate, force and conduction velocity
M_3	Bronchi, salivary glands, gastrointestinal tract, bladder, eye	Bronchoconstriction, gland secretion, increased gastrointestinal motility, sphincter dilation, bladder contraction, pupil dilation, vasodilation
M_4	Basal ganglia, prefrontal cortex, hippocampus	Enhanced locomotion
M_5	Substantia nigra, hippocampus	Cognition enhancement

Source: Rang & Dale, Pharmacology, 8th edition (2016); (Wess *et al.*, 2007)

The first ever studied parasympathomimetic substance was muscarin, isolated at the University of Tartu (Schmiedeberg & Koppe, 1869). The effects of typical antimuscarinics atropine and scopolamine used as herbal remedies have been known for centuries in the treatment of asthma, motion sickness and in analgesia. Arecoline, a partial cholinergic agonist and a psychoactive alkaloid naturally occurring in the betel nut, the fruit of *Areca catechu* palm, is mainly misused as a psychostimulant in South and Southeast Asia. Apart from the drug abuse, the anthelminthic and laxative effect are used in veterinary medicine (Volgin *et al.*, 2019). Pilocarpine is still used in eye drops to treat angle closure glaucoma and orally for inducing salivation in xerostomic patients (Ono *et al.*, 2018). All these substances are classical muscarinic ligands, used in the past till present (in various forms and derivatives), expressing the highly druggable potential of muscarinic receptors.

Advancements in medicinal chemistry and pharmacology enhanced the structure-based design and synthesis of selective drugs targeting mAchRs with better safety profile. However, the ongoing challenge for medicinal chemists in research for new potential pharmaceuticals is the true subtype-selectivity for muscarinic receptors. In many cases, the promising success of potential orthosteric drugs (mainly for the treatment of neurological diseases) in functional, binding studies and early phases of clinical trials was in later phases abandoned because of the undesirable side effects resulting from targeting peripheral M_2 and M_3 receptors. These include cardiovascular and gastrointestinal adverse reactions, which were reported with higher occurrence after administration of a studied drug (Felder *et al.*, 2018).

Publication of crystal structures of all of the known muscarinic receptors elucidated the mechanism of ligand binding on the molecular basis. Critical amino acid residues interacting with ligands were directly identified. All receptors were already crystallised bound with an antagonist, therefore in their inactive states. Additionally, M_2 active state structure and cocrystallised structure of bound agonist iperoxo and positive allosteric modulator LY2119620 were published, giving deeper insight into allostery and secondary binding sites (Kruse *et al*., 2013) (Vukovic *et al.*, 2019).

Amino acid residues interacting with ligands in the orthosteric binding pocket, when interacting with antagonists (3- Quinuclidinyl benzilate (QNB) or tiotropium) are very similar among all subtypes (Figure 1). Binding of tiotropium to M_1, M_3, M_4 and M_5 showed that the residues within the orthosteric pocket interacting with the antagonist are absolutely conserved among these subtypes. The spatial positions of extracellular loop 2 and extracellular loop 3 were subtly changed, which can lead to differences in ligand binding kinetics. Slight variations with different antagonists were observed, accounting for the relative subtype selectivity of the ligands but from this observation, it is clear that achieving absolute selectivity is hardly possible (Vukovic *et al.*, 2019).

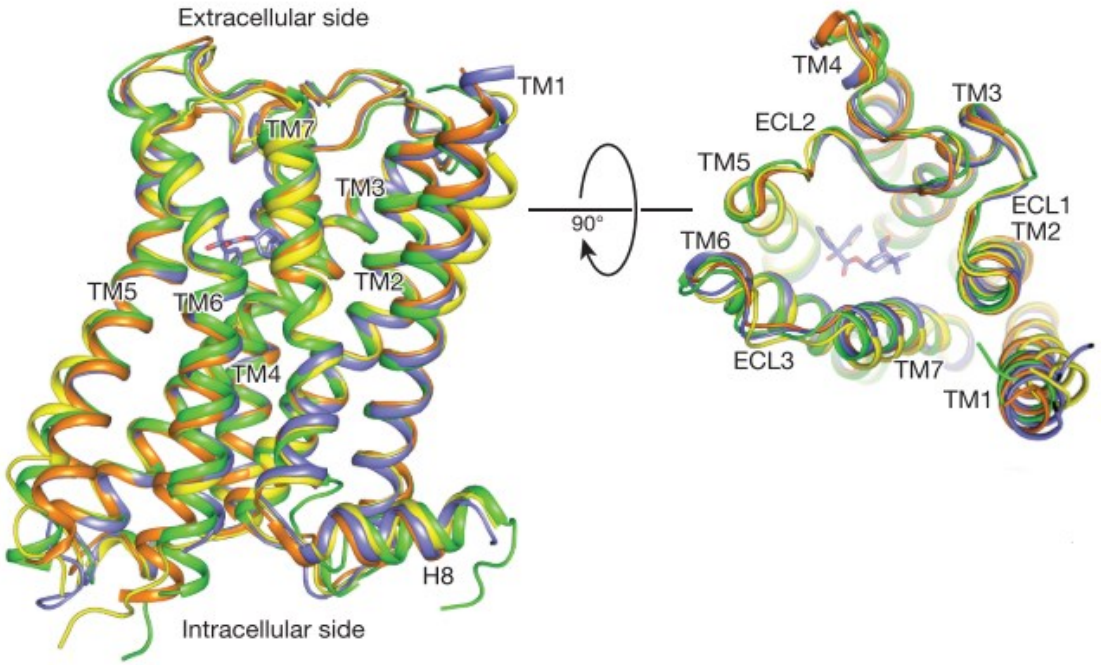

Figure 1 The structure similarity among M_1 – M_4 subtypes – the front view and the top down view on the binding pocket with antagonist tiotropium. M_1 depicted in green, M_2 in yellow, M_3 in orange and M_4 in blue. Modified from Thal (2016).

Each subtype has at least one allosteric binding site located in the extracellular vestibule above the orthosteric binding site, between the second and third extracellular loop. The structural heterogeneity in allosteric binding sites makes it possible to achieve subtype selectivity, making allosteric modulators suitable subjects for research. The current strategy is the synthesis and development of positive allosteric modulators (PAMs), allosteric agonists that can activate the receptor without the presence of an orthosteric ligand and bitopic ligands. (Burger *et al.*, 2018).

The modulation mechanism of PAM consists of maintaining the receptor in active, i.e., closed form after binding of an agonist, further stabilising the active conformation. Therapeutically, allosteric modulators have a ceiling effect – upon administration of increasing doses of PAM, the pharmacological effect reaches its maximum. Going beyond this concentration, the pharmacological effect remains unchanged and still with a low potential for occurrence of adverse effects (Burger, *et al.*, 2018).

In physiological conditions, PAMs can only function in presence of endogenous ligand. Bitopic ligands are composed of two structurally different pharmacophores binding

simultaneously to both binding sites (dualsteric binding). The orthosteric part (represented by classical ligand) is primarily responsible for receptor activation and allosteric part is navigating the former to the specific receptor subtype. These two parts are connected through a hydrocarbon chain spacer. They can achieve good efficacy and better selectivity compared to an orthosteric ligand itself. Hence their asset is that they don't require an endogenous ligand for the receptor activation. During the progression of neurodegenerative diseases, the level of Ach as endogenous ligand for mAChRs is gradually reduced, making this a valuable benefit (Felder *et al.*, 2018).

3.2.1. M_1 receptor

M_1 receptor is coupled with G_q protein, thus mainly mobilizing Ca^{2+} intracellularly through IP_3 and DAG pathway. It is referred to as the main muscarinic receptor of the CNS, expressed in many brain regions on postsynaptic neurons. The distribution predominates in the cortex, hippocampus (the main centre of memory formation), striatum and amygdala (Teal *et al.*, 2019).

The M_1-knockout (KO) mice experiments support the fact, that M_1 subtype (along with M_2 and M_5) is crucial for plasticity of neurons and cognition. Surprisingly, the KO mice did not show significant deficits compared to wild-type mice (in some tests, the memory was even enhanced) in most of the behavioural tests dealing with short-term memory. However, the KO mice did poorly in tests, where more complex task-solving was required. Concluded from these experiments, the M_1 receptors alone does not seem to be so important for memory formation but are more involved in consolidation and processes requiring the cooperativity of hippocampus and cortex (Anagnostaras *et al.*, 2002) .

The exact pathophysiology of Alzheimer's disease (AD) is not yet fully understood, with most of the explanations on the nature of the disease being hypotheses. The cholinergic theory supports the idea of progressive deterioration of basal cholinergic neurons in cortex, hippocampus and amygdala, leading to memory impairment. The theory was further supported by the observation, that cholinergic antagonists have negative effects on memory and learning and agonists enhance the memory formation. Interestingly, the number of postsynaptic M_1 receptors does not decrease during the progression of the disease, but they are apparently dysfunctional. Additionally, the activation cascade of this

receptor also supresses the deposition of β- amyloid peptide, one of the confirmed pathological features of Alzheimer's disease (Hampel *et al.*, 2018).

Cholinesterase inhibitors (donepezil, rivastigmine and galantamine) are predominantly prescribed in clinical practice to patients with mild to moderate symptoms of AD. They were developed in concordance with the cholinergic hypothesis, as their mechanism of action is the inhibition of acetylcholinesterase, the main enzyme responsible for acetylcholine hydrolysis. However, their ability to reverse the loss of cognition is modest and they tend to lose efficacy in many patients within the first year of administration (Knight et al., 2018).

Some promising muscarinic bitopic ligands, partial agonists and PAMs were already engaged in clinical trials on humans. Bitopic agonist LY593093, despite having high selectivity to M_1 over M_2 and M_3 *in vitro* and being well tolerated in subjects of research, did not reach over the 1st phase due to poor distribution to the brain. AZD6088, a M_1 partial agonist designed to treat neuropathic pain, did not proceed beyond 1st phase because of heart and gastrointestinal adverse reactions. A M_1 PAM MK7622 was used experimentally as an adjuvant therapy to cholinesterase inhibitor but turned out it lacked the effect on improving cognition in humans and caused higher rate of cholinergic adverse effects (Felder *et al.*, 2018).

These examples are mentioned to demonstrate that to register a new effective molecule with acceptable risk/benefit ratio is quite a challenge nowadays. Although there is a broad spectrum of sensitive assays and the computational molecular modelling have made a tremendous advance in past years, extrapolating preclinical data to clinical testing is still not perfect. The main problems that arose when testing ligands of this type on humans were predominantly ineffectiveness in studied indication (which can be the cause of low brain penetration and lack of biomarkers to evaluate it) and high occurrence of side effects most probably resulting from targeting peripheral muscarinic receptors (Felder *et al.*, 2018).

Latest study emphasizes the effect of biased signalling in response to adverse reactions. Two main M_1 signalling pathways – the G_q-protein and phosphorylation/arrestin mediated pathway were investigated on pathway-biased mouse models in terms of efficacy and potential to cause adverse effect. Concluded from the results, the M_1 G_q-

protein signalling pathway promotes the occurrence of cholinergic adverse reactions, that has been previously contributed to other muscarinic subtypes and the sole phosphorylation/arrestin activation is not reduced in the therapeutic potential. These findings give a new perspective to design of phosphorylation-biased muscarinic agonists and importance of biased signalling in general (Bradley et al., 2020)

3.2.2. M_2 receptor

The primary transduction mechanism is connected to G_i subtype leading to decrease in cAMP, thus mostly having inhibitory function. Predominantly, the M_2 is known for localization in heart atria, causing decrease in heart rate upon modulation of potassium channels via vagal stimulation. In the CNS, the expression is relatively uniform in all brain regions, a with higher distribution in cerebellum and spinal cord/pons and lower in cortex and hippocampus (Li *et al.*, 1991).

The effect to supress nocioception is contributed to M_2 receptors located in spinal cord. In wild-type mice, stimulation with agonist oxotremorine produced analgesic effect in tail-flick test. M_2 knockout mice showed reduced response to pain stimulation upon administration of oxotremorine and in double-knockout M_2/M_4 mice, the analgesic effect of agonist on pain tolerance was totally absent. Both receptor subtypes may therefore be interesting object for studies of novel analgesics (Wess *et al.*, 2007).

3.2.3. M_3 receptor

M_3 is G_q subtype receptor, mainly causing smooth muscle contraction on the periphery by activation of phospholipase C and releasing intracellular calcium from sarcoplasmatic reticulum.

In cooperation with the M_2 subtype, it causes contraction of bladder smooth muscle. The underlying mechanism is most likely by the extracellular Ca^{2+} influx and activation of rho kinase. Targeting bladder M_3 receptors is therapeutically used in treatment of overactive bladder, where antimuscarinics with preference to M_3 (and also M_2) such as solifenacin, darifenacin and trospium are widely used (Hegde *et al.*, 2006).

The activation of M_3 receptor in bronchi causes bronchoconstriction of the airway smooth muscle. Muscarinic antagonists classified according to duration of their action (slow- acting – SAMA and long-acting – LAMA) therefore mediate bronchodilation and

elevation of breathing. Ipratropium (SAMA) and newly also tiotropium (LAMA) are indicated for treatment of asthma in the form of dry-powder inhalers. LAMAs such as tiotropium, glycopyrronium and aclidinium all elevate the symptoms of chronic obstructive pulmonary disorder (COPD), dosed in inhalators or nebulizers. New potent drugs with long-acting profile and once-daily administration are being developed and tested (Hegde *et al.*, 2018).

Stimulation of peripheral M_3 receptors leads to exocrine gland secretion (salivary, sweat and lacrimal). Muscarinic agonists pilocarpine and cevimeline administered orally found its application in treatment of Sjögren's symptom - autoimmune condition, with symptoms of oral dryness and dry eyes originating from the absence of saliva and tears produced from exocrine glands (Siso, 2013).

M_3 receptor is a crucial cholinergic modulator of glucose-dependant insulin secretion from pancreatic β-cells. M_3-knockout mice are protected from insulin resistance and glucose intolerance because of lower fat deposits and higher level of fatty acid oxidation. Deficiency of these receptors is also connected to hyperactivity, higher energy expenditure and lower food intake (Gautam *et al.*, 2007).

Development of selective muscarinic agonists, PAMs and/or accessory proteins involved in the process of glucose homeostasis may be potential therapeuticals in treatment of glucose intolerance and type 2 diabetes. In a study with obese and lean mice, molecule VU0119498, PAM of acetylcholine, enhanced glucose induced insulin secretion and improved glucose tolerance (Zhu *et al.*, 2019).

3.2.4. M_4 receptor

M_4 is coupled with $G_{i/o}$ proteins, functioning mostly as inhibitory autoregulator of acetylcholine release in striatum, hippocampus and cerebral cortex and modulator of dopaminergic neurotransmission (Dencker et al., 2012).

When co-expressed with dopaminergic receptors on neurons of striatonigral pathway, they regulate the locomotor activity. Loss of dopaminergic neurons and disruption of this balance is a key factor in pathology of Parkinson's disease. Despite having many side-effects, central antimuscarinics such as biperiden and procyclidine are used in parkinsonian patients, relieving symptoms of involuntary movement and tremor.

Selective blockade of M_4 receptors in this brain area is therefore safer option for development of anti-parkinsonian drugs (Wess *et al.*, 2008). The potential analgesic effect upon stimulation in cooperation with M_2 subtype is already mentioned in the previous section.

3.2.5. M_5 receptor

The least explored type of the mAChRs is slowly gaining its significance thanks to publication of new information about its role in different brain regions. Among other tissues, it is expressed in endothelium of cerebral arteries, where upon activation it causes vasodilation followed by activation of NO-synthase. The M_5 - knockout mice showed reduced blood flow in many cerebral areas including hippocampus and basal ganglia. As a consequence, the progressive neuronal atrophy resulted in memory impairment and these mice showed poorer results in cognitive tests compared to wild-type mice (Wess *et al.*, 2008).

The presence of M_5 on postsynaptic dopaminergic neurons in ventral tegmental area and substantia nigra and their role in regulation of dopaminergic nigrostriatal pathway was recently studied with links to pathophysiology of schizophrenia (Bender *et al.*, 2019).

The role of M_5 in drug addiction pathophysiology is subject of extensive research based on the knowledge, that blocking of this receptor leads to inhibition of dopaminergic pathway (efflux of dopamine) in the nucleus accumbens, the important centre in the loop of processing reward stimuli. It appears, that substances based on negative allosteric modulation (NAMs) will have promising future as a medication in treatment of drug addiction (Bender *et al.,* 2019).

3.3. Mechanism of ligands binding to receptors

3.3.1. The law of mass action in pharmacology

The very basic and fundamental model of binding is described by the law of mass action. A free ligand binds to a free receptor with the association rate constant k_{on} forming a ligand-receptor complex. Since the reaction is reversible, the complex can break down to former units with the dissociation rate constant k_{off}. In this dynamic process, the equilibrium is reached when these rates are equal and the ratio of free ligand and free receptor to ligand-receptor complex is constant, described by an apparent dissociation constant K_d (Kenakin, 2015):

$$\frac{[Ligand]\,.\,[Receptor]}{[Complex\ Ligand\text{-}Receptor]} = \frac{k_{off}}{k_{on}} = K_d \quad (1)$$

A simple model like this has its limitations and it assumes the following:

- all receptors are equally accessible to the ligand
- the binding does not alter the receptor or ligand (in terms of receptor or ligand conformation)
- receptor is only in one affinity state (free or bound receptor), processes like partial agonism need more complex models
- The binding is reversible

This simple model is more than 150 years old and still used in many equilibrium binding assays to determine ligand binding affinities and ligand on- and off-rates. However, in this form and due to its restrictions, it fails to describe more complex processes (Kenakin, 2015).

3.3.2. Modes of ligand binding

Ligands can be divided into subgroups by their ability to stabilise a receptor in different conformations (activate or inactivate states) and initiate the response. Agonists and partial agonists first form an initial complex with the receptor, which then undergoes a transition into the active state, that allows binding of intracellular signalling proteins (mainly G- protein). Antagonists stabilise the receptor in the inactive conformations, lack the efficacy and hinder agonists from binding. Inverse agonists can reduce the receptor's basal activity (state when no ligand is present) therefore produce an effect opposite of the endogenous agonist (Latorraca *et al.,* 2017).

Mutant receptors with individually exchanged amino acid residues in the binding site are routinely used to illustrate critical ligand binding domains and nowadays, more than ever, go hand-in-hand with *in silico* methods. Thanks to advance in crystallography, spectroscopy and molecular dynamics modelling, ligand binding can be studied on atomic level (Latorraca *et al.*, 2017).

Muscarinic receptors are prototype receptors to study allosteric modulation. The common allosteric binding site is located in the vestibule of the orthosteric binding site, between the second and third extracellular loop. Conformationally, it is linked to the orthosteric

binding site. Allosteric binding of PAM causes narrowing of the vestibule and locks the orthosteric ligand in the binding pocket. The mechanism of this process varies in different muscarinic subtypes and is proposed as the key to PAM receptor subtype selectivity. More than one allosteric site has been identified among several muscarinic subtypes (Jakubik & El-fakahany, 2020). For instance, M_1 receptor possess a cryptic allosteric pocket not visible on crystal structure that binds only nonplanar M_1-selective PAMs (Hollingsworth *et al.*, 2019).

3.4. Methods to study binding to receptors

3.4.1. Fluorescence anisotropy assay

Fluorescent anisotropy assay is a method based on measuring the degree of polarization. This depends on the state of fluorescently labelled ligand (FL/tracer) in the solution. The tracer can be found either bound or unbound in the solution. Free tracer has more rotational freedom, therefore polarized light excites the spinning FL and results in depolarization of light and decrease in fluorescence anisotropy. On the other hand, ligand bound to receptor has less rotational speed, thus light emitted from the tracer-receptor complex will maintain the same plane of polarization, resulting in higher fluorescence anisotropy signal (Figure 2). To be exact, fluorescence polarization is only written in different mathematical form than FA and the measurement contains the same information. The term fluorescence anisotropy is more commonly used in biochemical and biophysical research. (Rinken *et al.,* 2018).

Fluorescence anisotropy in time *t* is calculated as:

$$FA\ (t) = \frac{I(t)_{\parallel} - I(t)_{\perp}}{I(t)_{\parallel} + 2 * I(t)_{\perp}} \quad (2)$$

Where $I(t)_{\parallel}$ corresponds to parallel fluorescence intensity and $I(t)_{\perp}$ corresponds to perpendicular fluorescence intensity, both at time *t*.

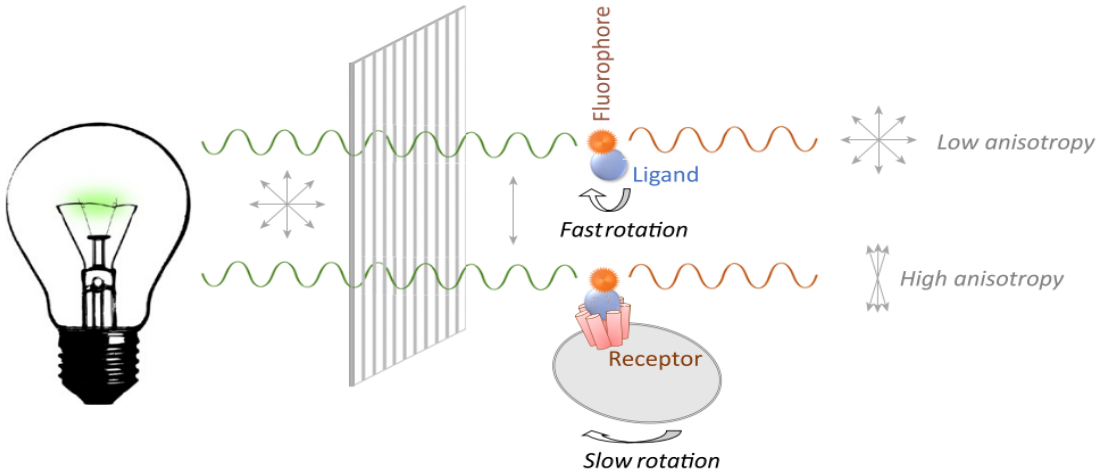

Figure 2 Mechanism of fluorescence anisotropy assay (Rinken *et al.*, 2018)

Concentration of both free and bound ligand must be taken into account, as the FA signal depends on both concentrations, which demonstrates the ratiometric principle of the assay. To measure the change in FA signal during binding, concentration of receptors and fluorescent ligand used must be in similar range (Rinken *et al*., 2018).

Most of the GPCRs ligands are not fluorescent by their nature and need coupling with a fluorescent moiety. Fluorescently labelled ligand generally consists of pharmacophore and fluorophore, connected directly together or conjugated via a linker. The pharmacophore can be an agonist or an antagonist (based on the nature of the experiment) with high affinity for the studied receptor. Since each fluorescent ligand is a unique pharmacological entity, affinity, efficacy and selectivity for each receptor subtype has to be well - specified. Both linker and fluorophore change ligands' properties, including interaction with the receptor. This is particularly true for low molecular weight molecules, where adding a bulky fluorophore and linker might lead to change in molecules' pharmacological, physiochemical or photophysical properties (Stoddart *et al.*, 2015).

Ideally, the fluorescent ligand should have affinity to the receptor in the low nanomolar or picomolar range. The ligand- fluorescent dye bond should be rigid enough to restrain the latter from free rotation. Length and composition of the linker must be optimised for each receptor and conjugated fluorophore, as it is in direct relation with the affinity (Baker *et al*., 2010). Also, water/lipid solubility of the fluorophore plays an important role in

inducing non-specific binding, e.g. to plastic surfaces or lipid membranes (Hughes *et al*., 2014).

When choosing suitable fluorophore, additional characteristics including fluorescence lifetime, fluorescence quantum and maximum absorption and emission wavelength must be evaluated. Fluorescence lifetime is the duration, in which the molecule remains in excited state, thus generates anisotropy. Fluorophores with lifetime of low ns area are ideal for the FA assay. Fluorescence quantum yield is the ratio of the number of emitted photons per absorbed. In FA assay, the fluorophore should have the ratio as high as possible, since it defines the detection limit of the assay (i.e. sensitivity in detection of quenching agents). Maximum absorption and emission wavelength should be high enough to prevent autofluorescence of the sample (Rinken *et al*., 2018).

Rising number of well-characterized fluorescent ligands for variety of assays and proteins (GPCRs, enzymes, nuclear receptors or ion channels) are becoming commercially available (e.g. BODIPY, Alexa series, rhodamine or fluorescein derivatives). Every assay requires specific and different characteristics of these molecules. One can choose from FL with lifetime, quantum yield, and absorption- emission wavelength and selectivity according to their needs (Iliopoulos- Tsoutsouvas *et al.*, 2018).

FA method represents homogenous, faster, sensitive enough and less expensive alternative to golden standard radioligand binding method. Fast screening for ligand kinetic parameters (association and dissociation rate constants and therefore equilibrium dissociation constant, half-life) and apparent affinity of the competitive ligand can be assessed by FA method. The requirements are that the competitor does not have significantly higher affinity towards the receptor than the fluorescent ligand. Additionally, apparent IC_{50} value cannot be lower than half of the total concentration of receptors used. Furthermore, due to the high signal-to-noise ratio and real time measurement of the assay, it is possible to use the experimental data to model more complex interactions occurring during the process of ligand binding, e.g. allosteric modulation or receptor oligomerisation (Rinken *et al.*, 2018).

3.4.2. Other fluorescent methods

Fluorescent spectroscopy methods present well-established techniques in GPCR studies. Their application varies from precise expression and localization of receptors in tissues

and cells, binding studies and inspection of complex molecular interactions involved with receptor conformational changes. With the use of confocal microscopy, the processes can be visualized over a timescale of several seconds, allowing real-time data analysis (Hern *et al.*, 2010).

Förster/fluorescence resonance energy transfer (FRET) is a method for detecting the interaction of molecules by spatial coincidence. In general, the fluorescently tagged donor passes the energy to acceptor with different fluorescent probe. The acceptor then emits a photon of specific wavelength, dependent on the nature of used fluorophore. The main requirements are overlapping emission spectra of donor and excitation spectra of the acceptor molecule and proximity of the donor and acceptor, which must be within 2- 10 nm. The dynamics of receptor-receptor or receptor-ligand interactions can be directly observed with FRET. Research based on allostery mechanisms, receptor dimerization and different types of agonism were conducted with the use of this method (Kauk & Hoffmann, 2017).

Total internal reflection fluorescence microscopy (TIRF-M) is an imaging method based on difference in refractive indexes of two different media. When the light hits the interface (usually glass slip-water) under critical angle, it becomes totally internally reflected and generates an evanescent wave. As the wave penetrates through the sample, it excites fluorophores in proximal region to the interface (within hundreds of nm). Since the wave decays exponentially with the distance from the interface, mainly fluorescent ligands bound to cell membranes are excited. Good signal to noise ratio and great resolution of this technique allows single molecule imaging and monitoring receptor dynamics (e.g. oligomerisation and internalisation) (Hern *et al.*, 2010; Stoddart *et al.*, 2015).

3.4.3. Radioligand binding assay

Radioligand binding assay has been a gold-standard method in quantitative studies of ligand binding. Radioisotope is incorporated into an unlabelled molecule, obtaining a radioactively labelled ligand, which must retain its selectivity and specificity of the unlabelled ligand for the studied protein. Wide variety of radiolabelled ligands is commercially available (Hulme & Trevethick, 2010).

Cells or membrane preparations are incubated with radioligand for required time, estimated as the time needed for reaching the equilibrium conditions. The crucial step is

the separation of bound and free radioactive ligand either by filtration or centrifugation including several washing steps. These procedures must be fast enough to avoid disturbance of equilibrium. The radioactivity is then measured on a scintillation counter, expressed as counts per minute (cpm) from the filter or pellet where bound ligand is present (Hulme & Trevethick, 2010).

This assay is widely used in ligand binding experiments, valued for its sensitivity when working with high affinity radioligands. Nevertheless, because of the separation and washing-out steps, the measurement dissociation kinetics becomes quite laborious, inflexible and lacking real-time information about the binding process, including very fast kinetic processes. Additional disadvantages are both cost of radiolabelled ligands and consumables for the assay, requirements for laboratories when working with radioactive substances and environmental issues and costs connected with radioactive waste disposal (Taylor, 2011).

3.5. Baculoviruses and Sf9 cells

Baculoviruses (BV) are double-stranded DNA viruses which infect insect cells (larvae of Lepidopteran species being the most common BV hosts). The genome consists of 80 – 180 kb and is packed into rod-shaped nucleocapsid. Dimensions of such particle are 30 – 70 nm in diameter and 200 – 40 nm in length. Occlusion-derived viruses (ODV) and budded viruses (BBV) are two phenotypes of enveloped virions. ODVs are responsible for the primal infection upon ingestion by a larvae and the envelope of these is acquired in the nucleus of the host cell. Infected cells then produce BV's, which spread the infection within one host and their envelope is derived from the plasma membrane of the host cells (Figure 3) (Okano *et al*., 2006).

For *in vitro* insect cells studies, BVs do not need the structural protein polyhedrin. Therefore, the cDNA for expression of desired protein can be subcloned into the vector under the control of polyhedrin promoter (although other promoters can be also used) and the gene for polyhedrin replaced with the gene for requested protein. Biosafety level 1 is required to work with BV's, since they are not infectious for humans (Martínez-solís & Herrero, 2019).

Baculoviruses present multipurpose gene delivery system, with its use ranging from pest control, vaccine development, gene therapy and tissue engineering to drug

screening (Mansouri & Berger, 2018). In FA assay, concentration of the fluorescent ligand and the receptor used must be in within the same range to see the course of the binding event. Therefore, high receptor density must be achieved with use of suitable overexpressing system (Rinken *et al.*, 2016). Baculoviruses serve both as suitable transfection vector for Sf9 cells as well as receptor carrier on their envelope derived from the plasma membrane of the infected insect cells (Figure 3).

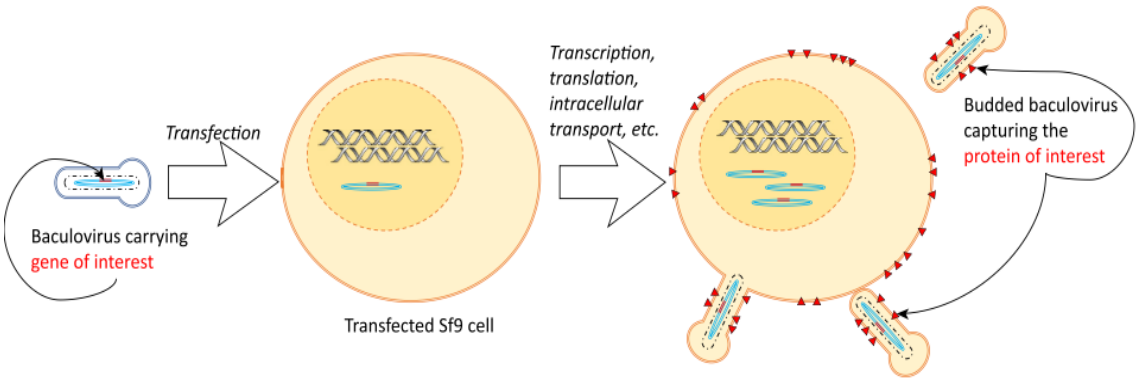

Figure 3 Production of baculovirus particles for FA assay (transferred from Rinken et al., 2018)

Whole cells overexpressing desired receptor, membrane preparations from such cells or solubilized receptors are commonly used for binding studies. Most widely used cell lines include Chinese hamster ovary (CHO) cells, Human embryonic kidney (HEK) cells and Sf9 cells. In comparison, BBVs provide low noise, low autofluorescence and homogenous system with stability in solution over time. Thanks to their small uniform structure, correct orientation of receptors in the mixture media and low sedimentation rate they serve as ideal alternative to predominantly used cell membrane preparations (Rinken et al., 2018).

Sf9 cells represent system extensively used in receptor binding and functional studies. These are insect cells derived from pupal ovarian tissue of the American fall army worm *Spodoptera frugiperda.* Successful transfection with mammalian gene of studied receptor encoded to viral vector is the key step for undergoing variety of assays. This cell line presents a suitable tool for characterization of GPCRs, thanks to its high expression level of recombinant proteins, post-translational modifications closely matching those in

mammalian cells and low endogenous constitutional receptor activity (Schneider & Seifert, 2010).

Although results from binding studies done with mammalian and insect cells are usually in good agreement, there are some physiological differences that must be taken into account when working with different cell lines and receptors (Lynch K., 1999; Massotte, 2003).

Mammalian cell membrane contains cholesterol, one of the main regulators of membrane fluidity (along with other phospholipids). Plasma membrane of insect cells is richer in unsaturated fatty acids and lacks cholesterol and sterols in general. However, the regulation of membrane fluidity was contributed to phosphatidylethanolamine, rather than cholesterol (Dawaliby et al., 2016). However, cholesterol possibly promotes cooperativity and oligomerization of different GPCRs (namely M_2, oxytocin or μ-opioid receptor). Ligands showed different affinity to receptors in Sf9 cell membranes treated with cholesterol compared to the native Sf9 cell membranes (Colozo *et al.*, 2007).

The other difference is simpler N-glycan processing in insect cells compared to mammalian. In the former, mannose terminates the oligosaccharide chain. In the latter, more complex N-glycan branches with mannose and galactose and terminated with sialic acid form the glycoprotein structure. This is due to lack of specific glycosyltransferases, enzymes catalysing transfer of galactose and sialic acid in mammalian cells (Hollister *et al*., 2002). To overcome this deficiency, 'humanized' Sf9 cells were bioengineered to produce mammalian-like N- glycans. Sialyation lead to improved pharmacokinetic properties (extended circulatory lifetime), higher stability and immunotolerance of bioengineered biopharmaceuticals (Solá & Griebenow, 2010).

4. Aim

The aims of this thesis are characterization of ligand binding properties to M_1 mAChR in budded baculoviruses with fluorescence anisotropy assay using TAMRA-labelled MK342 as reporter ligand.

The study includes:

- Construction of budded baculoviruses expressing M_1 mAChR
- Validation of the FA method to study ligand binding to receptors
- Determination of binding affinity of MK342, FA values of receptor-ligand complex and non-specific binding, the expression level of the M_1 receptor
- Determination of apparent affinities of fourteen different muscarinic ligands

5. Experimental part

5.1. Materials and methods

5.1.1. Modified Krebs-Ringer buffer

Composition of buffer (Table 3): 135 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 11 mM Na- Hepes pH = 7.4 (1 M stock solution was prepared by dissolving HEPES free acid (Amresco Inc., Ohio) in ultrapure water (Millipore Milli-Q). 10% NaOH was added to adjust pH = 7.4), 0.1 % Pluronic F-127 (Invitrogen) (granulated Pluronic was dissolved in ultrapure water in ratio 1:10 by thoroughly shaking in incubator), 25x Complete EDTA- free Protease Inhibitor Cocktail (Sigma- Aldrich) (stock solution prepared by dissolving 1 tablet in 2 ml of ultrapure water).

Fresh buffer was prepared for each experiment and stock solutions of chemicals mentioned above were diluted with ultrapure water to listed concentrations for the assay.

Table 3 Composition of modified Krebs-Ringer buffer

Solution	Concentration
NaCl	135 mM
$CaCl_2$	1 mM
KCl	5 mM
$MgCl_2$	1 mM
Na-HEPES	11 mM
Complete EDTA-free protease inhibitor cocktail	25x
Pluronic F-127	0,1%

5.1.2. Cell culture

Spodoptera frugiperda Sf9 cells (Invitrogen Life Technologies) isolated from pupal ovarian tissue of the fall armyworm were used for cell transfection and baculovirus amplification. Cells are grown in suspension culture with EX-CELL® 420 Serum-Free Medium (Sigma-Aldrich) for insect cells. The optimal conditions for cell growth are in non-humidified environment, 27°C, 0% CO_2 atmosphere in polypropylene Erlenmayer flasks on rotary shaker (~110 rpm), kept at density 1-4x10^6 cells/ml.

For each cell culture experiment, density and viability of cells was determined by following procedure: 10 µl of cells suspension and 10 µl of 0.4% trypan blue (Sigma- Aldrich) was mixed in Eppendorf tube. 10 µl of preparation was transferred on both sides of dual-chamber slide and inserted into TC10^{TM} automated cell counter (Bio-Rad Laboratiories AB, Sundbyberg, Sweden). The average cell density and viability was calculated by averaging four measurements (two measurements from each chamber).

5.1.3. Stock solution of baculovirus

cDNA (Invitrogen, Missouri-Rolla University cDNA Resource Centre) of the wild type M_1 receptor was previously subcloned to restriction site of the pFastBac1 vector by Dr. Anni Allikalt and the initial recombinant baculovirus stock was prepared following the same protocol as described in cited publication (Veiksina *et al.*, 2015).

5.1.4. Infection of Sf9 cells and virus amplification

To obtain large volume of BV particles to study ligand binding, cells have to be infected with high-titre baculovirus stock solution at low multiplicity of infection (MOI). The volume of baculovirus stock solution needed for infection is calculated as:

$$\mathrm{V(virus)} = \frac{\mathrm{MOI} * \mathrm{V(medium)} * \text{cell density}}{\text{virus titer}} \qquad (3)$$

In this case, to obtain 110 ml of transfected Sf9 cells, cell culture at density $1.5x10^6$ cells/ml was infected with 18 µl of budded baculovirus stock solution of previously determined high-titre at MOI=0.01. Amplification is considered successful after cell viability drops below 50%, therefore cell growth and viability must be screened for subsequent days. 80 hours post-infection, cell viability was 35%, hence virus supernatant was collected. Suspension was pipetted to 50 ml conical centrifuge Falcon tubes and centrifuged at 3000 g for 10 minutes to remove cells and cell membranes. Virus supernatant was harvested and stored at 4°C until the virus concentration was determined.

5.1.5. Determination of virus titre by Image-based cell-size estimation assay

There are many different methods to quantify virus concentration – antibody-based, cell viability or plaque forming assays (Roldão, Oliveira, Carrondo, & Alves, 2009) just to name few. Each having its limitation, Laasfeld *et al.* developed assay and ICSE-Tools software based on change in cell diameter upon infection with virus particle using

bright- field microscopy (Laasfeld *et al.*,2017). ISCE Tools is an open source software available at http://www.gpcr.ut.ee/software.html.

Cells were counted and diluted to concentration $8x10^5$/ml with EX-CELL®. 250 µl of cell suspension was transferred to each well of BioLite 24-well plate (Thermo-Fisher Scientific). The plate was kept in an incubator for 20 minutes and after this period, examined under microscope to check cell attachment to the plate. Baculovirus solution was prepared in three-fold dilutions (from 2x dilution to 118098x dilution) in 12 Eppendorf tubes in total volume 600 µl for each tube. 250 µl of each virus dilution was transferred in duplicates to each well with attached cells. Blank wells contained only cells and EX- CELL®. Prior to image analysis, the plate was incubated for 24 h at 27°C for the infection to take place.

The images were captured using Cytation™ 5 Cell Imaging Multi-Mode Reader (Bio-Tek Instruments) equipped with Gen5 software. Four images per well were taken and analysed with ICSE-Tools software. Briefly, the ICSE-tools converts bright-field images to binary images and from these calculates average cell diameter (Figure 4) Obtained values were fitted using non-linear regression dose-response curve and the concentration of virus was calculated with following equation:

$$ivp/ml = \frac{N}{2 * EC_{50} * V} \tag{4}$$

where N represents number of cells per well, EC_{50} represents virus concentration, at which the average cell diameter changed by 50% and V is volume in millilitres per well.

Concentration of the prepared baculovirus stock solution was $9x10^7$ infectious viral particles in millilitre. As this concentration is sufficiently high, produced virus solution was used for transfection.

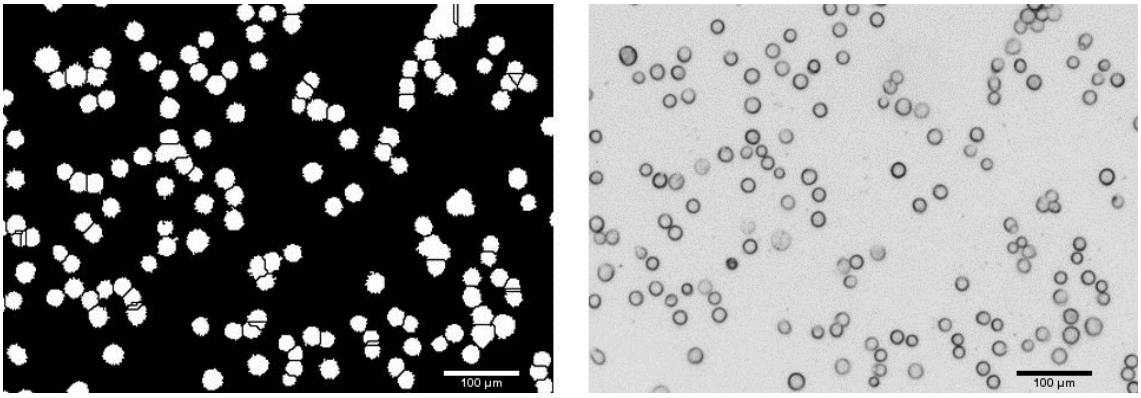

Figure 4 Binary image (left) and brightfield image (right) of transfected Sf9 cells from Cytation 5

5.1.6. Transfection of Sf9 cells and budded baculovirus preparation

Cell suspension at density $2x10^6$/ml was transfected with 100 ml of produced high-titre baculovirus stock solution at MOI 3.5 (previously optimized for baculoviruses encoding M_1 receptor). Eighty hours post-infection, cell viability had fallen under 20%. Cell suspension was centrifuged at 3000 rpm for 10 minutes and the virus supernatant was collected.

Baculovirus suspension was centrifuged at 48 000 g, 4°C for 40 minutes on Sigma 3K30 and Sigma 3-30KS centrifuge (Sigma Laborzentrifugen). Supernatant was discharged and the formed BV pellet was washed with 700 µl of cold buffer and afterwards resuspended in 500 µl of cold buffer to obtain 50x concentrated budded baculovirus preparation. Samples were aliquoted and stored at - 80°C. Two batches of budded baculovirus preparation were prepared separately within 2 months period.

5.1.7. Fluorescence anisotropy measurements

Fluorescence anisotropy measurement was done on Synergy Neo Microplate Reader (BioTek Instruments) with 530 nm excitation and 590 nm emission filter. Gains were set upon calibration with Eryhrosine B as a standard. Experiments were undertaken in 96- well black opaque flat bottom microplate (Corning). Total solution volume in one well was 100 µl. Budded baculovirus preparation was always added in the last step to initiate the binding reaction. Custom-made lid was used to prevent evaporation. Fluorescence anisotropy (FA) at time *t* was calculated from (2).

All measurements were blank-corrected for background fluorescence by subtracting the FA value of the well containing only buffer with BBV preparation (without fluorescent ligand) from FA of all the other wells.

5.1.8. Fluorescent ligand

Fluorescent ligand MK342 (Figure 5) used for this assay is tetramethylrhodamine (TAMRA) labelled dibenzodiazepinone derivate, specific to muscarinic receptors. It was provided by Dr. Keller (University of Regensburg, Germany). Stock solutions were diluted in dimethyl sulfoxide (DMSO) and stored at -20°C, protected from the light.

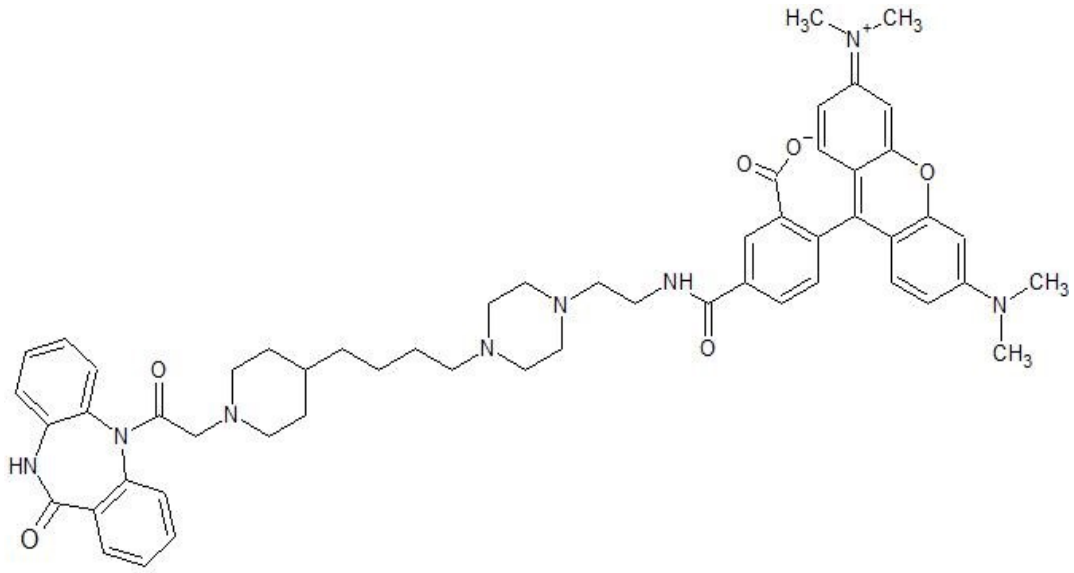

Figure 5 Structure of fluorescent ligand MK342

5.1.9. Determination of receptor concentration and dissociation constant of the fluorescent ligand

2-fold dilutions of prepared budded baculovirus stock solutions were diluted on a separate Axygen® clear round bottom 96-Well 2 ml polypropylene deep well plate (Corning). Wells for total binding contained 30 µl buffer, 30 µl of fluorescent ligand (1 nM or 5nM) and 40 µl of corresponding dilution of baculovirus preparation. Non- specific binding was measured with 30 µl scopolamine (final concentration 1 µM), 30 µl of fluorescent ligand (1 nM or 5 nM) and 40 µl of corresponding dilution of baculovirus preparation.

Concentration of M_1 receptors displayed on BBV particles was calculated from receptor stock concentration by the following equation:

$$[R]_T = [R]_{stock} * \frac{V_{BV}}{V_{well}} \quad (5)$$

where $[R]_t$ is total receptor concentration in the well, $[R]_{stock}$ represents receptor stock concentration calculated from globally fitted data from (Veiksina *et al.*, 2014) V_{BV} is the volume of the baculovirus preparation in the well and V_{well} is total volume in the well.

5.1.10. Competitive binding assay

Eleven classical muscarinic acetylcholine receptor ligands were tested, namely 4- DAMP (Research Biochemicals Incorporated), acetylcholine (Tocris Bioscience), arecoline (Sigma-Aldrich), atropine (Merck), carbachol (Tocris Bioscience), McN-A-343 (Tocris Bioscience), N-methylscopolamine (Sigma-Aldrich), pilocarpine (Tocris Bioscience), pirenzepine (Sigma-Aldrich), scopolamine (Sigma-Aldrich) and telenzepine (Research Biochemicals Incorporated).

Three dibenzodiazepinone-type bitopic M_2-selective ligands UR-MK259 (monomeric), UR- SK- 59 (heterodimeric) and UR-SK-75 (heterodimeric) were provided by Dr. Keller (University of Regensburg, Germany). Initial stock solutions were stored in DMSO at - 20°C.

First, 8-point serial dilutions of non-labelled competitive ligands in the buffer were prepared with dilution factor 6 or 8. 30 µl of the fixed 5 nM concentration of MK342 were added to each well, except for the blank samples. The reaction was initiated by the addition of 20 µl of baculovirus preparation (for the first batch) or 40 µl of baculovirus preparation (for the second batch). Total binding was determined in the absence of the competitive ligand, in wells containing only buffer, 5 nM fluorescent ligand and 20 µl or 40 µl baculovirus preparation. Measurement in the microplate reader was done at temperature 27°C.

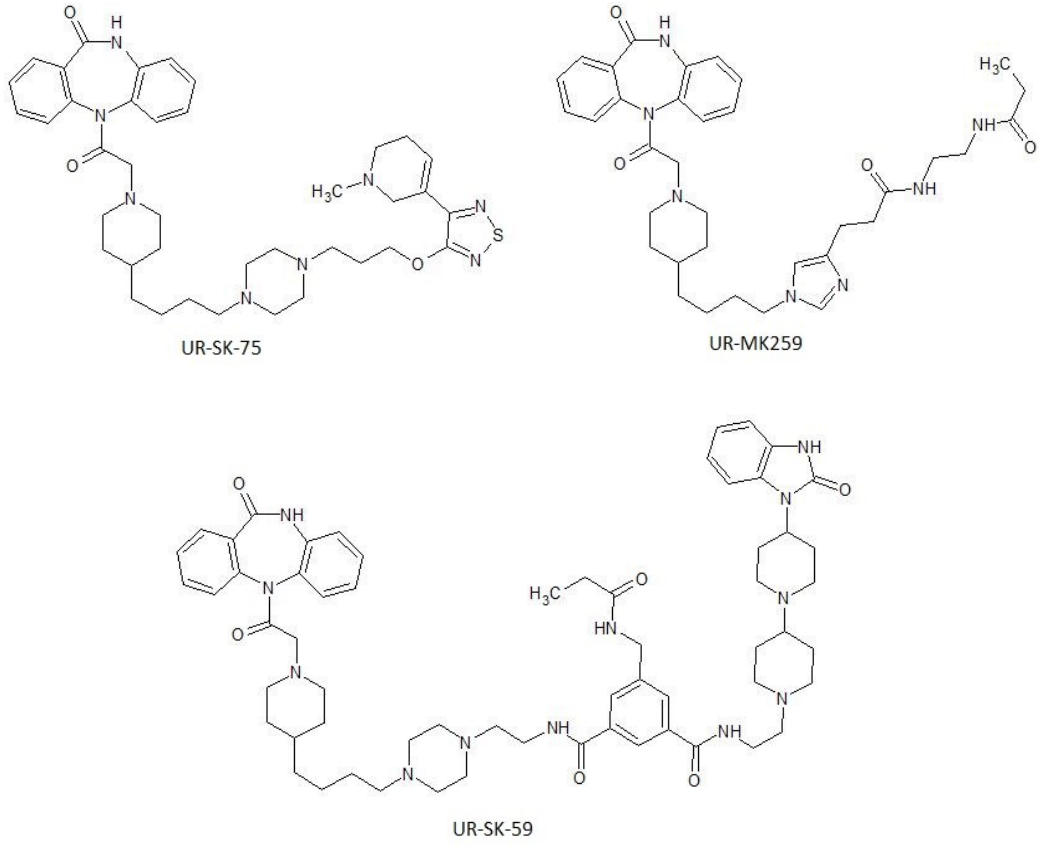

Figure 6 Structures of M_2-selective dibenzoazepinone derivatives

5.2. Data analysis

Optimization of experimental data (blank correction, time correction, experimental setup etc.) prior to GraphPad Prism analysis was done with open source software Aparecium 2.0, developed by Tõnis Laasfeld available at http://www.gpcr.ut.ee/aparecium.html

Data were analysed with GraphPad Prism (version 5.00 for Windows, GraphPad Software, La Jolla California USA).

To take into the account ligand depletion and non-specific interactions, measurement of K_d and concentration of the M_1 receptors in BBV solution were globally fitted to set of user-defined equations. Details are described in Veiksina *et al.*, 2014.

Apparent potency expressed as $\log IC_{50}$ was calculated using dose-response curve:

$$FA = FA_{min} + \frac{FA_{max} - FA_{min}}{1 + 10^{\,logC - logIC_{50}}} \tag{6}$$

Where FA_{max} corresponds to highest anisotropy value, FA_{min} is the lowest anisotropy value, $logC$ is the logarithm of concentration of competitor and $logIC_{50}$ is the logarithm of concentration of the competitor causing 50% of fluorescence anisotropy change of the fluorescent ligand.

The Cheng-Prusoff equation (7) was used to calculate the pKi from the pIC_{50}.

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}} \tag{7}$$

Where K_i is the inhibition constant, IC_{50} is the concentration of the competitor causing 50% of fluorescence anisotropy change of the fluorescent ligand, $[L]$ is the concentration of fluorescent ligand used and K_d is the equilibrium dissociation constant of the fluorescent ligand.

As this calculation method has limitations for the FA assay, the extra uncertainty of 0,2 calculated from modelling theoretical data was added to the standard error of the mean (S.E.M.). This is in results section stated as S.E.M with correction.

6. Results

6.1. Determination of binding affinity of MK342 and concentration of M_1 mAChR in BBV preparation

Addition of increasing concentrations of BBV preparation expressing M_1 receptors to MK342 lead to significant change in the fluorescent anisotropy with signal window of around 0,054 anisotropy units for specific binding (Figure 7). FA of the free fluorescent ligand (both 1 nM and 5 nM concentrations without BBV preparation) in the buffer was around 0,02 anisotropy units. As the K_d for MK342 was already measured earlier by Dr.Allikalt (4,4 nM), this value was used for calculation of receptor concentrations in the preparations using global fitting equations (Veiksina *et al.*, 2014) and constraining the K_d parameter. The obtained receptor concentrations after 5 hours incubation period for the first batch of BBV preparation was 53 ± 8 nM and for the second batch 24 ± 11 nM.

Total receptor concentration was calculated from Eq. 5. Maximal relative specific binding compared to non-specific binding was achieved at 20 µl for the first batch of BBV preparation, calculated concentration of M_1 receptor was 11 nM. Expression of M_1 receptors in the second batch was lower, maximal relative specific binding compared to non-specific binding was achieved at 40 µl of added baculovirus preparation meaning M_1 receptor concentration 10 nM (Figure 7). These volumes with receptor concentration were therefore used in competition binding experiments.

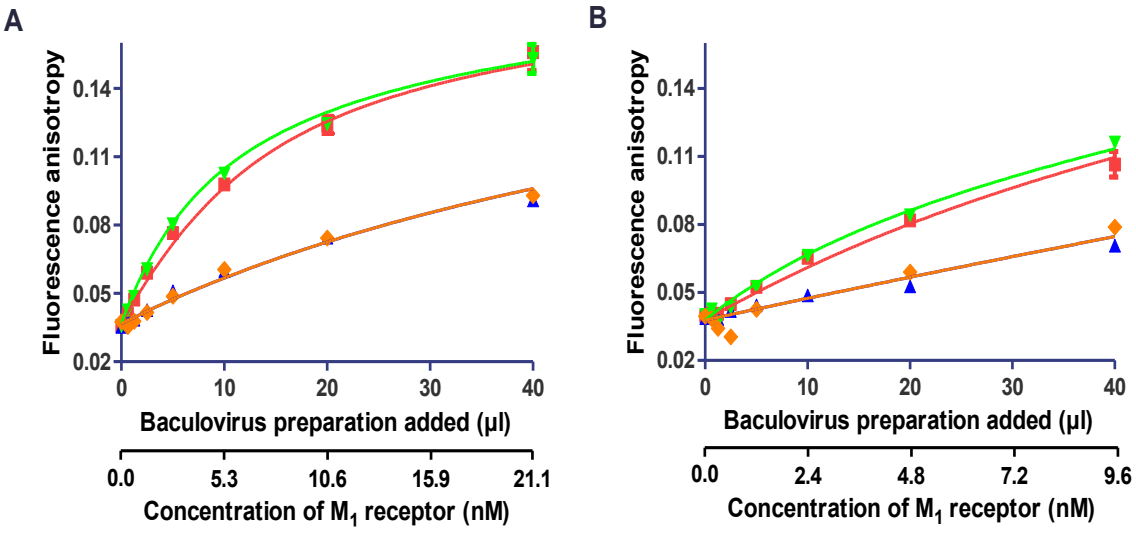

Figure 7 Binding curves of the first (A) and the second (B) batch of the M_1 receptor expressing baculovirus particles dilutions with fluorescent ligand MK342. FA of total binding was measured after 5 hours of incubation with 1 nM (▼) and 5 nM (■) of MK342. Non-specific binding measured in the presence of 1 µM scopolamine, MK342 concentrations were 1 nM (◆) and 5 nM (▲). Receptor concentration was calculated after the fitting of the experimental data. Data presented as mean ± SD after 5 hours of incubation from one experiment done in duplicates.

6.2. Competitive binding

6.2.1. Classical muscarinic ligands

From eleven studied classical muscarinic ligands, only carbachol did not produce a typical competitive curve with MK342. Maximal concentration of carbachol used (10 mM) inhibited MK342 binding to maximum 30%. In the first 40 minutes of the reaction, fast rise of anisotropy (in extent of 0,009 anisotropy units) was observed in the highest concentration of carbachol (10 mM) compared to the lower concentration and absence of carbachol. However, we do not know if it is due to fast binding of the tracer enhanced by carbachol or formation of aggregates in such high concentrations of the competitor.

Summary of competitive binding results (apparent pIC_{50} values and calculated pK_i) are in Table 4. A linear correlation between pK_i values from the FA assay and pK_i values previously published in the literature was found (Figure 9). The coefficient of determination was $R^2 = 0,95$.

Table 4 The pIC_{50} values of muscarinic ligands for M_1 mAChR determined in competition with 5 nM MK342 and calculated pK_i* in comparison with previously published pK_i from International Union of Pharmacology (IUPHAR) Guide to Pharmacology (telenzepine from (Boer & Eltze, 1989). $_pIC_{50}$ values are from three independent measurements done in duplicates.

Ligand name	pIC_{50} ± S.E.M.	Calculated pK_i ± S.E.M. with correction	Previously published pK_i
Agonists			
Acetylcholine	2,73 ± 0,18	3,06 ± 0,38	4,3 – 4,9
Arecoline	3,36 ± 0,19	3,69 ± 0,39	5,7
Carbachol	Not detected		3,2 – 5,3
McN-A-343	3,23 ± 0,11	3,56 ± 0,31	4,8 – 5,2
Pilocarpine	3,67 ± 0,18	4,00 ± 0,38	5,1
Antagonists			
4-DAMP	7,24 ± 0,08	7,57 ± 0,28	9,2
Atropine	7,77 ± 0,05	8,10 ± 0,25	8,5 – 9,6
NMS	7,66 ± 0,19	8,00 ± 0,39	9,9
Pirenzepine	6,47 ± 0,09	6,80 ± 0,29	7,8 – 7,9
Scopolamine	8,43 ± 0,11	8,76 ± 0,31	9,0
Telenzepine	7,04 ± 0,18	7,37 ± 0,38	9,0

*pK_i were calculated from IC_{50} according to the Cheng-Prusoff equation (Eq. 7). K_d value of 4,4 nM was used for the tracer

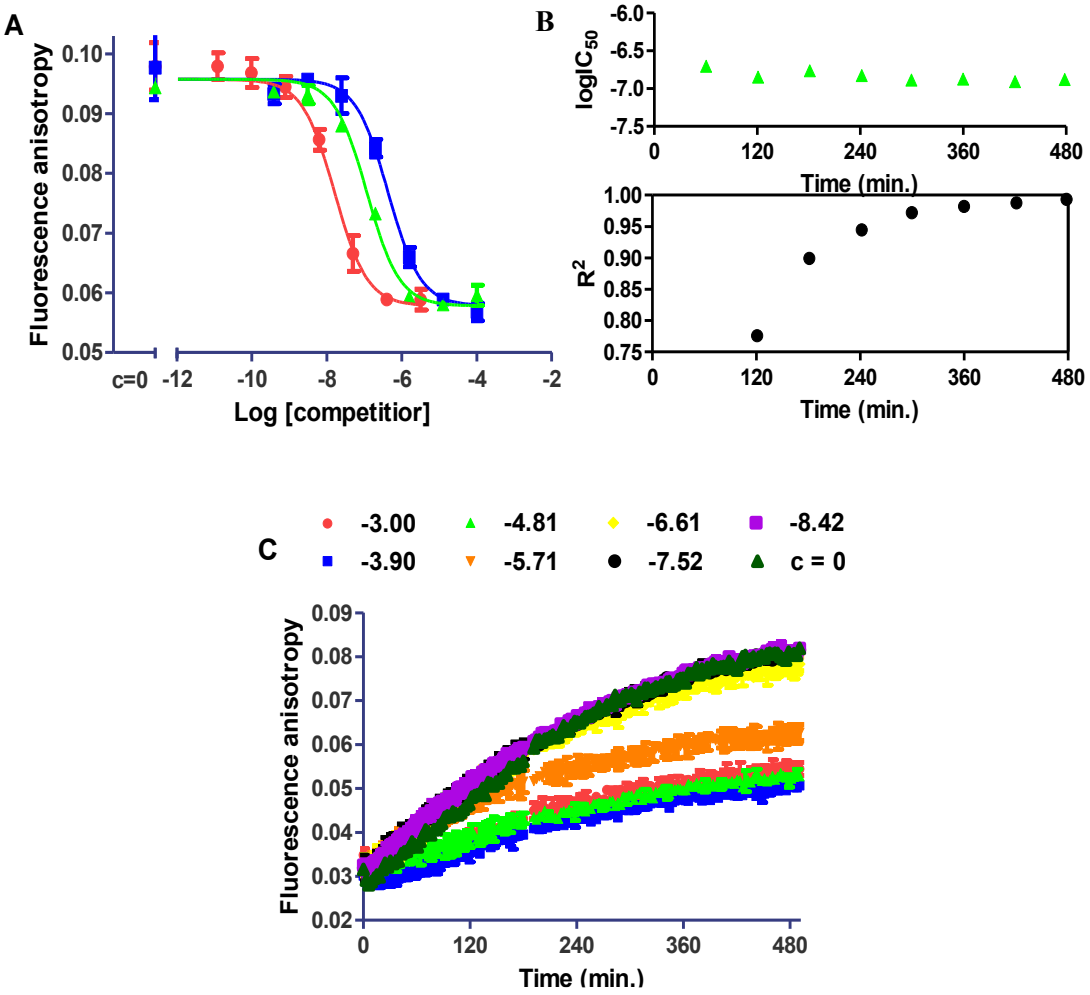

Figure 8 Competition of muscarinic antagonists with fluorescent ligand MK342 binding M_1 receptor (**A**) Different concentrations of atropine (red ●), telenzepine (▲) and pirenzepine (■) were incubated for 8 hours at 27°C with fluorescent ligand MK342 (5 nM) in presence of budded baculoviruses expressing M_1 receptor (**B**) Change and stabilization of $logIC_{50}$ of telenzepine in time compared to R^2 (identical experimental conditions as in (A)). (**C**) Association binding of the fluorescent ligand MK342 to M_1 receptor in the budded baculovirus preparation with different serial dilutions of competitive ligand telenzepine. Concentrations of telenzepine are indicated in the graph in the logarithmic scale. The data presented as mean ± SD are from a representative experiment from three independent measurements done in duplicates.

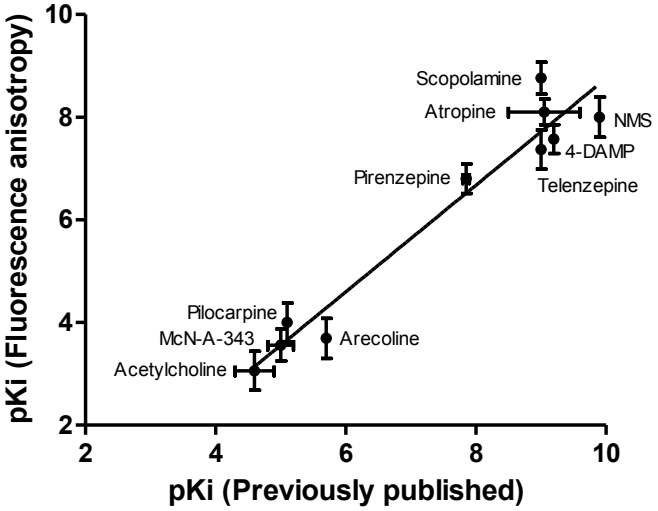

Figure 9 Comparison of apparent affinities (pK_i) of classical muscarinic ligands measured in FA with previously published pK_i. $R^2 = 0,95$. Error bars indicate mean ± S.E.M. with correction

6.2.2. Bitopic M_2-selective muscarinic ligands

All three bitopic ligands caused concentration-dependent decrease in FA values of the fluorescent ligand. Potencies of UR-SK-59 and UR-SK-75 were in good agreement with previously published radioligand binding data with [^{3}H]NMS. The apparent affinity of UR-MK259 measured in the fluorescent anisotropy assay with MK342 was almost 2 orders of magnitude lower than already reported from the radioligand binding (Table 5). However, when comparing displacement curves of these three ligands, we discovered that UR-SK-59 and UR-SK- 75 are not able to displace the fluorescent ligand to the same extent as UR-MK259 (difference in anisotropy of the highest concentration of UR-SK- 59 and UR-SK-75 compared to UR- MK259 being approximately 0,017 anisotropy units) (Figure 10).

Table 5 Apparent affinities (pIC_{50}) and calculated pK_i^* of bitopic competitors in comparison with pK_i from She et al., 2017. Results are from number of measurements stated as N done in duplicates.

Ligand name	pIC_{50} ± S.E.M.	Calculated pKi* ± S.E.M. with correction	Previously published pK_i	N
UR-MK259	5,93 ± 0,40	6,25 ± 0,60	8,07	2
UR-SK-59	8,25 ± 0,09	8,58 ± 0,29	8,56	3
UR-SK-75	8,06 ± 0,10	8,39 ± 0,30	8,84	3

*pK_i were calculated from IC_{50} according to the Cheng-Prusoff equation (Eq. 7). K_d value of 4,4 nM was used for the tracer

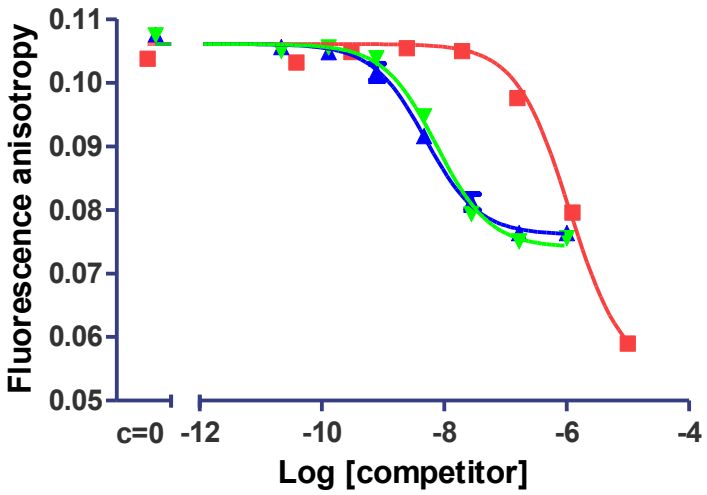

Figure 10 Competitive binding with fluorescent ligand MK342 to M_1 receptor expressed on budded baculovirus particles. 8-point dilutions of competitors UR–SK-59 (blue ▲), UR- SK-75 (green ▼) and UR-MK259 (red ■) were incubated for 10 hours at 27°C. Data presented as mean ± SD are from a representative experiment from three indenpendent measurements done in duplicates.

Binding of MK342 can be observed on-line without disruption of the reaction. We compared binding of the tracer with these three ligands in time. In presence of 1 μM UR- SK-59, 1 μM UR- SK- 75 and 1,25 μM UR-MK259 from the beginning of the measurement, the fluorescence anisotropy was already higher than in other concentrations of the competitor by approximately 0,02 anisotropy units and then remained stable over time (Figure 11).

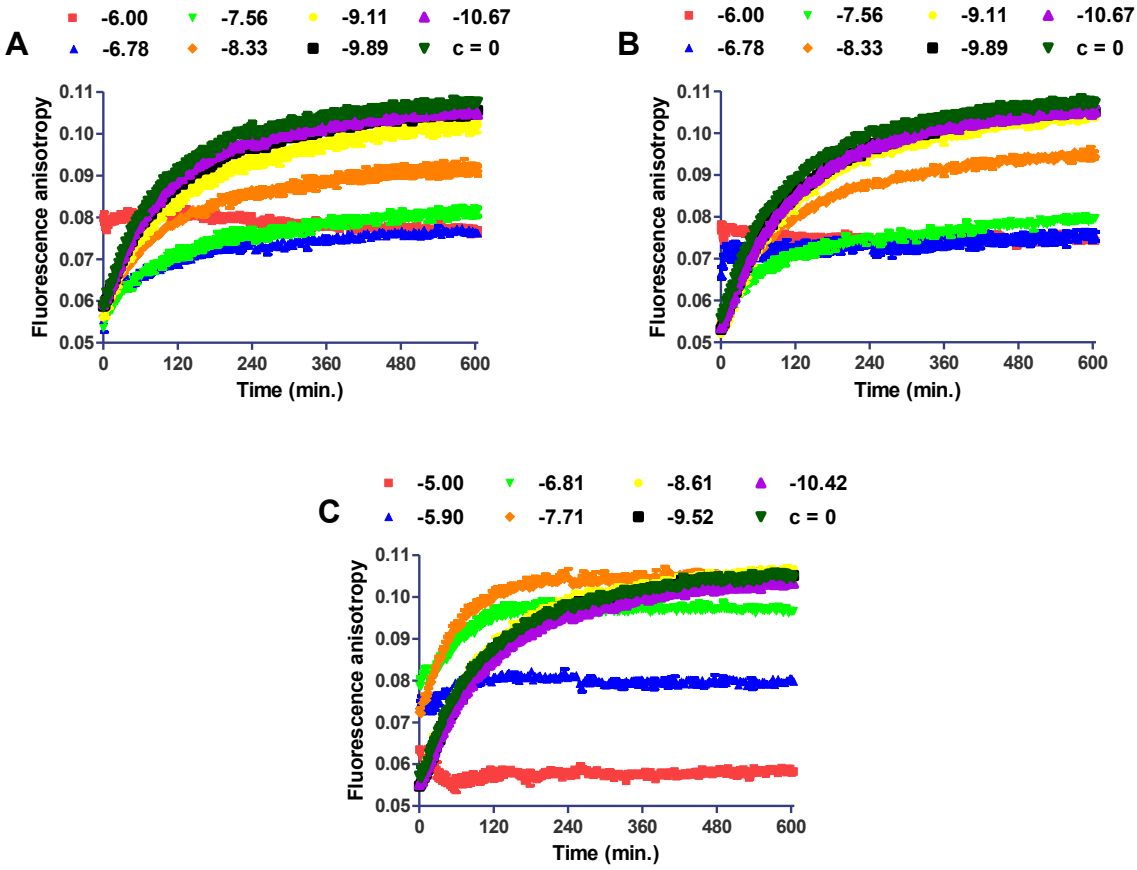

Figure 11 Association binding of the fluorescent ligand MK342 to M_1 receptor in the budded baculovirus preparation with serial dilutions of competitive ligands **UR-SK-59 (A)**, **UR-SK-75 (B)** and **UR-MK259 (C)**. 8-point dilutions of competitors with 5 nM MK342 were incubated for 10 hours at 27°C with budded baculovirus preparation. Concentrations of competitors are indicated in graphs in the logarithmic scale. Presented data are from a representative experiment from three independent measurements done in duplicates.

To investigate this abnormal binding, dissociation experiment with UR-MK259 was done. Association of 5 nM MK342 to M_1 receptors was after 4 hours followed by dissociation with 1 μM and 10 μM concentrations of UR-MK259. Non-specific binding was detected in presence of 10 μM UR-MK259 with 5 nM MK342 and M_1- receptor in

BBV preparation. A time-dependent decrease in FA values indicated dissociation of fluorescent ligand from the receptor-FL complex. The fluorescent ligand did not fully dissociate (did not reach the level of non-specific binding) from the receptor-bound complex in presence of both 1 μM and 10 μM concentrations of UR-MK259 (Figure 12).

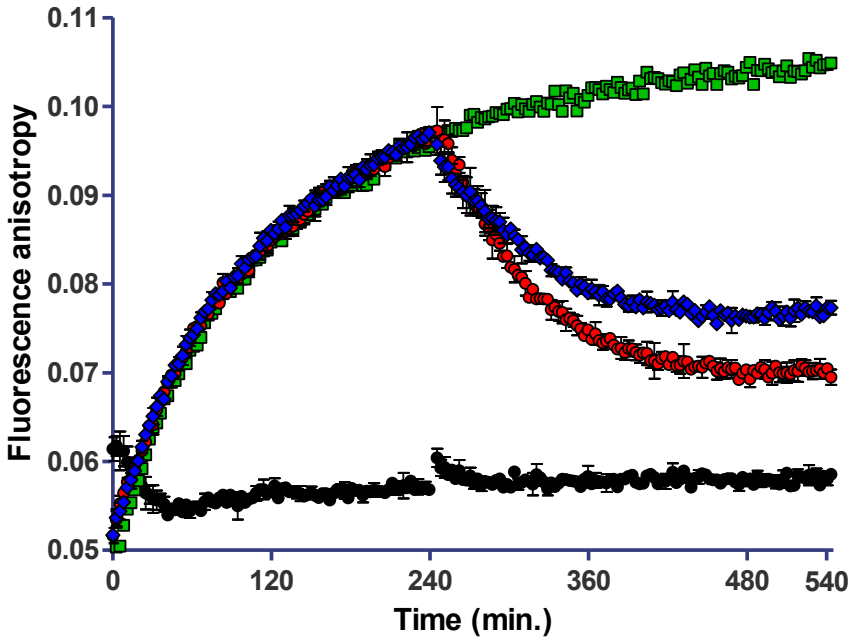

Figure 12 Time course of anisotropy change (association and dissociation) caused by fluorescent ligand MK342 binding to M_1 receptors in BBV preparation. 5 nM MK342 was incubated for 4 hours with M_1 receptor in presence (●) and absence (green □, blue ◇ and red ○) of 10 μM UR-MK259. Dissociation was initiated after 4 hours by addition of 10 μM (red ○) and 1 μM (blue ◇) UR-MK259. The reaction took in total 9 hours. Data presented are from a single experiment done in duplicates, data are presented as mean ± SD. Experiment was repeated one more time with similar result, dissociation was initiated with 10 μM UR-MK259 only.

7. Discussion

Muscarinic M_1 receptors represent a vital part in the human physiology, with main distribution in the central nervous system. Their role in pathophysiology of neurodegenerative diseases is widely studied which makes them attractive targets for novel drug development (Felder et al., 2018). The studies of ligands binding in preliminary drug development is routinely assessed with radioligand binding. As an alternative, fluorescence anisotropy is a method to study the mechanism of ligand binding with use of fluorescently labelled ligands, where binding event can be studied in real time without any further separation steps required (Hoffmann et al., 2015).

In this study, we used fluorescent ligand MK342 selective to muscarinic receptors to determine its binding to M_1 muscarinic receptor expressed in budded baculovirus particles and study its displacement in competitive binding studies.

The binding of MK342 to M_1 mAChR in BBV caused FA change with signal window of approximately 0,04 anisotropy units. It was therefore possible to use it as a probe for receptor characterization and use for screening for apparent affinities (pIC_{50}) of competitive ligands. From in total fourteen substances, pIC_{50} of thirteen competitors was obtained. Only pIC_{50} of carbachol was not determined because the maximal concentration we were able to use was not high enough to displace the florescent ligand from the receptor-bound complex.

Although a good correlation between our data and already published data and was found, the apparent pIC_{50} value of classical ligands was in most cases lower than that previously published, for agonists was the difference from 1,7-2,3 orders of magnitude, in case of antagonists 0,6-2.2 orders of magnitude. This can be attributed many factors including different expression system, different concentration of studied receptors than in the published literature, different kinetics of used tracer and also dynamics of the system.

The concentration of M_1 receptor in the competition assay is approximately 10 nM. The low limit of IC_{50} is in this case 5 nM, because the apparent IC_{50} cannot be lower than half of the total receptor concentration used. Low limit of IC_{50} is also set by the K_d of the tracer, in our case 4,4 nM. Generally, the higher is the tracer K_d, the wider range of affinities of competitors can be resolved (Huang, 2003). Although the higher

concentration of receptors is connected with higher anisotropy signal (higher dynamic range), it also results in higher IC_{50} (Owicki, 2000).

Use of a high expression system is crucial for this type of assay. Although budded baculoviruses present a homogenous system with low noise, their membrane derived from Sf9 cells are very poor in cholesterol, which is present in mammalian cells. In radioligand binding assay in the case of M_2-receptors, cholesterol-treated Sf9 cells promoted cooperativity in the binding of radiolabelled antagonists and moderately enhanced affinity of NMS compared to native Sf9 cells (Colozo *et al.*, 2007). Also, G_q -proteins expressed in *Sf9* cells often inefficiently interact with GPCRs (Schneider & Seifert, 2010) and some authors speculate on higher affinity of some agonists to M_1 receptor in G-protein-coupled state (Huwiler et al., 2010). Screening for binding affinities in radioligand binding assay done on Sf9 cells, HEK293 cells and budded baculovirus particles expressing dopamine D_1 receptor showed higher affinities of agonist binding to the receptor in HEK293 (system expressing G-proteins) compared to Sf9 and BBV particles (Allikalt & Rinken, 2017). It is questionable, to what extent would be the binding affected in M_1 receptors by use of different expression system.

Based on the additional 40% decrease in anisotropy in the case of 10 μM concentration of UR-MK-259 compared to the other bitopic ligands, it can be proposed that the fluorescent ligand MK342 might be a dualsteric ligand, binding to both orthostetric and allosteric binding site and 10 μM concentration of UR-MK259 can fully displace it from both binding sites.

In case of UR-MK259, UR-SK-59 and UR-SK-75, rapid rise of anisotropy signal in higher concentration of competitors can be observed. The bitopic competitors may at specific concentration enhance the binding affinity of the tracer to the allosteric binding site on a fraction of receptors.

In the dissociation experiment with 1 μM and 10 μM concentrations of bitopic competitor UR-MK259, the fluorescent ligand was partially irreversibly bound to the receptor. It can be speculated that in the concentration added for dissociation, UR-MK259 may occupy the allosteric binding site that leads to conformation where the fluorescent ligand is blocked from dissociation from the orthosteric binding site or vice versa.

All experiments were analysed using one-site binding model, which does not suppose more states than free fluorescent ligand, free competitor, fluorescent ligand bound to receptor, competitor bound to receptor and non-specific binding. We propose that competitive binding of MK342 with bitopic ligands is more complex process and different model is needed to to fully explain molecular dynamics which are occurring during the binding process. We can assume that the MK342 is a unique dualsteric tracer for M_1 mAChR suitable for screening of both orthosteric and bitopic ligands. These results can be further analysed with more complex models to study ligand binding into more detail and propose additional hypotheses. Docking could also elucidate the binding mechanism of both the tracer and bitopic ligands.

8. Conclusion

In this experimental work we studied binding of fluorescent ligand MK342 to muscarinic acetylcholine receptor subtype 1 expressed on the membrane of budded baculovirus particles using fluorescence anisotropy method. Our preparations of budded baculovirus particles had high enough expression of M_1 receptors to study ligand binding. Fluorescent ligand MK342 was specifically bound to M_1 receptor with high affinity and could be used as a tracer in competitive binding studies. Eleven classical muscarinic ligands and three bitopic ligands were screened for their binding potencies (apparent pIC_{50} value) and compared to already published data. The pharmacology profile of the studied ligands corresponded to the profile found earlier for M_1 mAChR, while their apparent affinities were lower. Nevertheless, good correlation ($R^2 = 0,95$) of obtained and published pK_i value was found. Based on the competitive binding with bitopic ligands, we additionally propose that the tracer can be a dualsteric ligand making it possible to study bitopic ligands.

More experiments with MK342 on M_1 receptors should be done to define its kinetic parameters and also study the role of allosteric modulators on ligand binding. FA studies to determine affinity of this fluorescent ligand towards other subtypes of muscarinic receptors can be performed to employ its possible applicability in wider spectrum of targets. Results from this work are a step ahead in studies of ligand binding mechanisms to mAChR and further studies are required e.g. with molecular modelling to better understand molecular dynamics and characterize ligand binding.

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