Chapter 3

Review 2 – Small Molecule Fluorescent Ligands as Central Nervous System Imaging Probes

Article to be submitted as a mini-review for publication

ChemMedChem

Small Molecule Fluorescent Ligands as Central Nervous System Imaging Probes

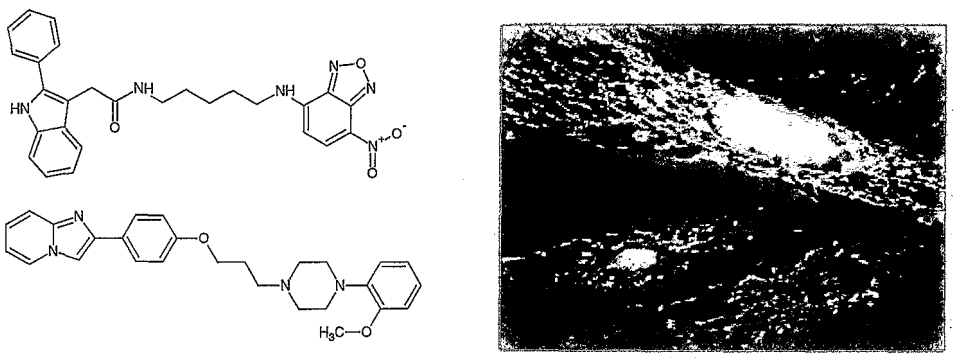
Jacques Joubert[1,2], Prof. Sandra v Dyk[2], Prof. Sarel F Malan[1*]

[1] *School of Pharmacy, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.*

[2]*Pharmaceutical Chemistry, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.*

*Corresponding author at present address: School of Pharmacy, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa. Tel: +27 21959 3190; fax: +27 21959 1588; e-mail: sfmalan@uwc.ac.za

Graphical Abstract



The design and development of small molecule fluorescent ligands as imaging probes for neurological targets have been under intense investigation in recent years. This review will focus on recent advances in the design of small molecule fluorescent ligands and sophisticated imaging techniques utilised to effectively visualize these target molecules.

Abstract

The design, development and use of small molecule fluorescent ligands to directly or indirectly study receptors, enzymes and other targets in the central nervous system (CNS) have in recent years become an intense area of investigation, especially for use in quantitative, sensitive and direct binding assays to study target proteins, both intra- and extra-cellularly and as prodromal diagnostic tools. The rapid development of ultra sensitive fluorescent spectroscopic approaches, such as fluorescence correlation spectroscopy, flow cytometry, confocal laser scanning microscopy, fluorescence polarization and multi-photon fluorescence microscopy, is opening new scenarios for the use of small molecule fluorescent ligands in the study of CNS pharmacology. In combination with efficient labeling protocols, these techniques offer enormous possibilities at the micro- and nanometer level to develop parallel multifaceted tools in pharmacological sciences. This review covers small molecule fluorescent ligands that have been applied to study proteins and other targets in the CNS through visualization by means of fluorescent imaging technologies.

Keywords: Fluorescence, Fluorescence Spectroscopy, High-Throughput Screening, Imaging Agents, Neurochemistry.

3.1. Introduction

Radioligand binding techniques and receptor gene assays have been widely used to study the pharmacology and physiology of receptors, enzymes and other target proteins in the CNS.[1-3] These methodologies provide an efficient means for drug discovery and identification of putative endogenous substances that may physiologically subserve various target proteins in the brain, including amongst others receptors and enzymes.[3,4] These studies are particularly useful in characterising drug-target protein interactions and specificities. The understanding of drug and CNS receptor and enzyme interactions has been greatly advanced by especially radioligand binding techniques.[4] There are numerous examples in literature describing the development and use of radioligand binding techniques. For example, Snyder *et al.*, described the characterisation of numerous drugs and their interactions with calcium channels and benzodiazepine receptors by means of radioligand binding studies.[5] Olsen *et al.*, described the characterization of $GABA_A$ receptor subtypes using autoradiographic comparison of GABA, benzodiazipine, and convulsant binding sites in the murine CNS.[6] Wulfkuhle *et al.*, and Leung described classical radiographic assays involving radioactively labelled drug

candidates or lipids, allowing estimates of protein-ligand interactions in the CNS.[7,8] Despite the usefulness and sensitivity of radioligand binding techniques, the use of alternative methods to study ligand-protein interactions, especially in the CNS, may provide information not readily accessible by conventional radioreceptor techniques and circumvent some of the drawbacks, such as high cost, disposal, speed, health hazard and potential technical implications, associated with this methodology.[1-4] A major bottleneck in the discovery of new drugs is the availability of fast, effective, efficient, easy and safe screening methods.[3]

Fluorescent ligands have been used since the mid 1970s and represent a safer, faster, and less-expensive alternative to traditional radioligand binding techniques.[2,3] The use of fluorescent ligands is growing rapidly within the pharmacological and related communities and has proven to be valuable for the investigation of interactions of different CNS proteins with their ligands in complement to classical methods such as radioligand binding and site-directed mutagenesis.[9,10] In the past, fluorescent ligands were used in the same way as histological stains with little attention being paid to the concentrations being used. There has however been a growing realisation that a number of factors can influence the final location and binding of a fluorescent drug and that previously, these factors have been ignored.[3] From a pharmacological viewpoint, it is essential that the affinity and selectivity of the fluorescent drug be known. This should be defined not only in recombinant cells but also at the native receptors and on the tissues that are to be studied.[4] Unfortunately, commercial sources often, if at all, provide only affinity values from radioligand binding studies of cell membranes harvested from cells that have been programmed to overexpress a particular protein.[3] Fluorescent ligands with high specificity and attractive spectroscopic properties could add significant value in the field of biomedical research. It is therefore an object of this review to discuss small molecule fluorescent ligands for use in directly quantifying ligand-protein interactions, intracellularly and extracellularly, and as prodromal diagnostic tools.

Fluorescent based technologies, such as fluorescence correlation spectroscopy, flow cytometry, confocal laser scanning microscopy (CLSM), fluorescence polarization and multi-photon fluorescence microscopy (MPFM), offers an alternative and adds a valuable supplement to classical methods such as radioligand binding and site-directed mutagenesis. These techniques offer extremely sensitive, fast, noninvasive, nonradioactive, insight into the local environment of the fluorescent ligand and the possibility to obtain on-line information on the interactions studied.[1-4] In addition, fluorescent ligands, if appropriately designed,

present the added advantage of retaining the pharmacological profile of the parent, unlabeled ligand, thus allowing localization and real time monitoring of processes, such as internalization, trafficking, sequestration, recycling and other thermodynamic and kinetic parameters, triggered by ligand-protein interactions in living cells.[11,12]

Fluorescent high-affinity ligand-bound receptors, enzymes or other proteins in the CNS can show their location and, if binding is reversible, can provide pharmacological information such as affinity and proximity between interacting molecules. Fluorescent ligands thus represent a class of widely applicable tools and may provide information on the mechanism of ligand binding,[12,13] the movement and internalization of receptors and enzymes in living cells,[14,15] the physical nature of the binding pocket(s)[16,17] and visualization of labeled proteins using fluorescent imaging techniques.[18,19] Consequently, the relationships between the location, function and life cycles of various CNS protein targets have become better understood as a result of fluorescent labeling.[3] Some environmentally sensitive fluorescent ligands can also give information on the biophysical characteristics of the ligand binding site because their quantum yield (brightness) is dependent on the lipophilicity and/or pH of the environment.[20,21] As a further advantage, fluorescent ligands can also be competitively displaced from their binding site(s) of a target protein by non-fluorescent ligands, allowing the identification of the sites recognized by the ligands.[1,2] This represents the possibility of developing high-throughput screening (HTS) assays utilizing fluorescent ligands. Each of these aspects may contribute to new insights is drug action and potential new drug targets for CNS disorders.[3,22]

The use of small molecule fluorescent ligands in combination with fluorescence techniques to probe the structure and function of membrane proteins has proven in recent year to be extremely powerful method with numerous applications for CNS imaging ranging from biophysical characterization of the ligand site and ligand interactions to fluorescent imaging of membrane proteins in living cells.[3,23-25] This review will focus on some of these applications but is not intended to give an exhaustive list of all fluorescent ligands developed for CNS imaging assays, but rather to focus on selected examples in literature highlighting advances and potential application(s) of this methodology.

3.2. Basic principle of fluorescence

Fluorescence is a luminescence that is mostly found as an optical phenomenon in cold bodies where energy is supplied by an external source such as an incandescent lamp or a laser and absorbed by a fluorescent molecule.[23] Fluorescence is the result of a 3 stage process; (1) excitation, (2) excited state lifetime, and (3) fluorescence emission (Figure 1).[3,24-25] This process occurs in certain molecules, generally polyaromatic hydrocarbons or heterocycles, called fluorophores.[23] There is a loss of energy between excitation and emission causing the emitted photon to have a lower energy and thus longer wavelength than that of the excited state.[3,25] Singlet and triplet states refer to the energy level of a chromophore. Absorption of a photon causes a transition to an excited singlet state that decays to a corresponding triplet state. When an excited fluorophore is raised to a singlet state, it decays back to ground state in a variety of ways.[3,23-25] The most common way is emission of a photon that generates a fluorescent signal. However, powerful excitation can raise the fluorophore to a triplet state that undergoes a chemical interaction with molecular oxygen to generate a nonfluorescent molecule and cause photobleaching.[26,27] Addition of free radical scavengers, also known as anti-fade agents, can reduce the rate of photobleaching.[3] Competing with emission is a number of other mechanisms which are capable of depopulating the singlet state namely; collisional quenching,[3,26,27] fluorescence resonance energy transfer (FRET)[23] and intersystem crossings.[26-28] These quenching effects are measured as the fluorophores quantum yield and many of the sensitive fluorescent techniques used with fluorophores are negatively affected if the fluorophores are subject to these quenching mechanisms.[26-28] Therefore, careful choice of the fluorophore that will be conjugated to the biological ligand and an understanding of the spectral properties of the molecule and imaging techniques to be used are of the utmost importance.

As a general rule, a fluorescent molecule excited at a given wavelength emits light at a longer wavelength. This phenomenon is described as the red or Stokes shift and allows the user to detect the emitted light over the background of the light source used to excite the fluorophore.[3,26] In contrast, absorption spectrophotometry requires measurements of transmitted light relative to high indent light levels at the same wavelength. Stokes shift is often referred to in the literature when describing the fluorescence properties of a molecule.[23]

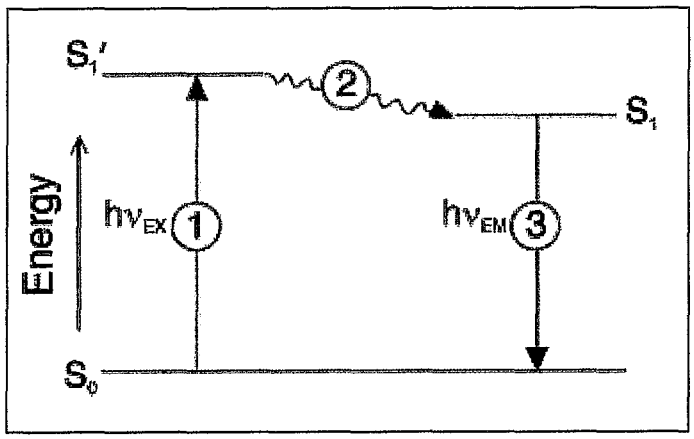


Figure 1: The Jablonski diagram illustrating the processes involved for emission of fluorescence. At room temperature, molecules are predominantly present in the lowest vibrational level of the electronic ground state, S_0. By absorbing light (energy) of the correct specific wavelength, a molecule can be excited (1) to any of the vibrational levels of the first excited electronic state S_1. The excited fluorophore will then lose energy and relax to the lowest vibrational level (2) of the excited state S_1, from which it returns to any of the vibrational levels (3) of the ground state S_0 thereby emitting a photon (fluorescence).[23]

3.3. Choice of Fluorescent labels (Fluorophores)

The design of a small molecule fluorescent ligand for CNS imaging starts from the selection of the appropriate fluorophore to be conjugated with the biologically active ligand. The basic criteria for selecting an appropriate fluorophore are: (i) It may not hinder binding by the ligand to the active site of the target-protein, i.e., the specificity and selectivity of the ligand to only one receptor or enzyme; (ii) It must be detectable in the visible spectrum and; (iii) It should not affect the biological activity of the ligand.[2,3] Although this is the basic criteria for the design of a fluorescent ligand, there are some exceptions to this rule as will be discussed later. Appropriate positional attachment of fluorophores to biologically active ligands is critical to retain both binding affinity and efficacy. In this respect, the knowledge of structure–activity relationships is crucial to the development of fluorescent ligands. Yates *et al.* conjugated the fluorescent dye, nitrobenzodiazole (NBD), directly to the cannabinoid CB2 receptor agonist, JWH-015, but abolished the biological activity of the resulting molecule.[29] This loss of pharmacological activity may, in some cases, be prevented by separating the pharmacophore (active part of the parent ligand) from the fluorescent moiety through the introduction of a spacer into an area of the molecule that has minimal influence on biological activity. For example, Balboni *et al.* described the synthesis, opioid activity profile and fluorescent parameters of a H-Dmt-Tic-Glu-NH2 analogue.[30] Fluorescein was linked through a pentamethylene spacer at the C-terminus because N-terminus basicity is fundamental for opioid activity. The use of structure-activity knowledge led to the discovery

of a fluorescent ligand for the opioid receptor. Another group used structure activity relationships identified from previous studies to develop 3-phenyltropane fluorescent ligands for the dopamine transporter (DAT) retaining activity.[31] Optimal affinity of the ligands for the DAT was achieved by attaching an ethyl linker to the 2-position of the tropane ring to which the amine-reactive fluorophore, rhodamine red-X NHS ester, was conjugated. The compound retained activity and could be effectively used as a fluorescent ligand for the DAT. Spacer length should ideally result from an optimization process. Numerous studies have reported systematic evaluation of spacer length for the fluorescent probe under study.[32,33] One such study by Joubert *et al.* described the synthesis of a series of adamantane fluorescent ligands with *N*-methyl-D-aspartate (NMDA) receptor channel activity where the increase in chain length compared to direct conjugation of the fluorophores resulted in a significant increase in activity.[34] The authors concluded that a longer linkage allowed the adamantane moiety to reach deeper sites in the putative binding sites of the NMDA receptor, resulting in increased activity.

Additional factors to consider when selecting a fluorophore for CNS imaging is the spectroscopic behavior thereof and whether or not it will emit detectable fluorescence at the desired wavelength(s) under the conditions the fluorophore is intended to be detected i.e. pH, ionic strength, polarity and the tissue present.[33] Environment-sensitive fluorophores have attracted much attention in this regards because of their spectroscopic behavior, which is dependent on the physicochemical properties of the surrounding environment. Particularly useful are the solvatochromic fluorophores that display sensitivity to the polarity of the local environment such as 4-dimethylaminophthalimide, 4-amino-1,8-naphthalimide and dansyl.[35] These molecules generally exhibit low quantum yield in aqueous solution but become highly fluorescent in nonpolar solvents or when bound to a hydrophobic site in proteins or membranes.[33,35,36]

Another aspect to consider in the design of effective CNS fluorescent ligands, especially those to be used in *in vivo* imaging protocols, such as prodromal diagnostic tools for neurological disorders, is the ability of the ligand to permeate the blood-brain barrier (BBB).[37] Small molecule fluorescent ligands with a molecular weight of less than 600 requires a moderate degree of hydrophobicity to effectively cross the BBB into the CNS. However, as ligands become more hydrophobic, they may be retained longer in the brain and may show higher nonspecific binding for hydrophobic structures. Thus, there is an inherent trade-off between

brain biodistribution and non-specific background contrast.[38] This presents a major challenge in the design of both *in vitro* and especially *in vivo* CNS fluorescent ligands.

Examples of small molecule fluorescent labels (fluorophores) that have been successfully used in the design of fluorescent ligands as CNS probes include, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), dansyl chloride, anthranilic acid, BODIPY, coumarin, texas red, fluorescein, cyanoisoindole and rhodamine.[3,11,23,39] These molecules are suitable to be used in the design of CNS fluorescent ligands since they are relatively small molecules that would not be expected to sterically hinder a ligand-protein interaction if coupled to a parent molecule at an appropriate position using a suitable spacer group and exhibit favorable spectrofluorometric properties for CNS imaging. However, the structural modification associated with the inclusion of the fluorophore can change lipophilicity, affinity, and selectivity or even change a competitive ligand into a noncompetitive ligand and this always have be taken into account when designing CNS fluorescent ligands.[33,40]

3.4. Fluorescent ligands as imaging probes for CNS targets

Various CNS targets exist for the development of drugs as therapeutic agents against neurological disorders. This has presented researchers with the opportunity to design imaging technologies to aid in the drug design and discovery process by optimizing the process through fluorescent techniques, either by developing HTS methods or to gain a deeper understanding of the target under investigation. There have been numerous reports in recent years on the design of suitable fluorescent ligands for CNS target imaging, some of which are discussed below.

The adenosine A1-receptor is responsible for mediating the physiological effects of adenosine in diverse tissues such as the brain, heart, kidney, and adipocytes.[41] Research has shown that this receptor in the CNS may be a useful therapeutic target against various neurodegenerative disorders.[42] Recent development of fluorescent ligands to study this receptor and its involvement in neurodegenerative disorders have been presented by Middleton *et al.*[32] This group reported the identification of fluorescent adenosine A1 receptor agonists derived from the adenosine analogue *N*-ethyl carboxamide, NECA. Previous structure–activity relationship studies indicated that modifications at the *N*6-position of the NECA analogue could retain activity. This allowed for the insertion of *N*6-aminoalkyl linkers of various lengths to yield the corresponding functionalized congeners that were labelled with the fluorophore BODIPY.

Despite a tripling of molecular weight compared to the parent agonist, all of the molecules displayed potent competitive agonism at the A1 receptor. One of these agonists, **1**, was effectively used to visualize, by CLSM, the A1 receptor on CHO cells.

In addition to this study another group also reported a potential fluorescent ligand, 4-[6-(dansylamino)hexylamino]-7-methyl-2-phenyl-1,8-naphthyridine (**2**), for studying adenosine A1-receptors.[43] This compound preserved, or even slightly improved, the high affinity (13 nM) for the A1 receptor subtype shown by its non-fluorescent naphthyridine counterpart (17 nM). Moreover, the dansylated naphthyridine (**2**) showed an A1/A2 selectivity ratio which is four to five times better than that found with non-dansylated naphthyridine. The binding properties of compound **2**, together with its structural simplicity and ease of synthesis, makes it a promising and convenient fluorescent ligand for *in vitro* localization and trafficking studies of the A1 adenosine receptor.

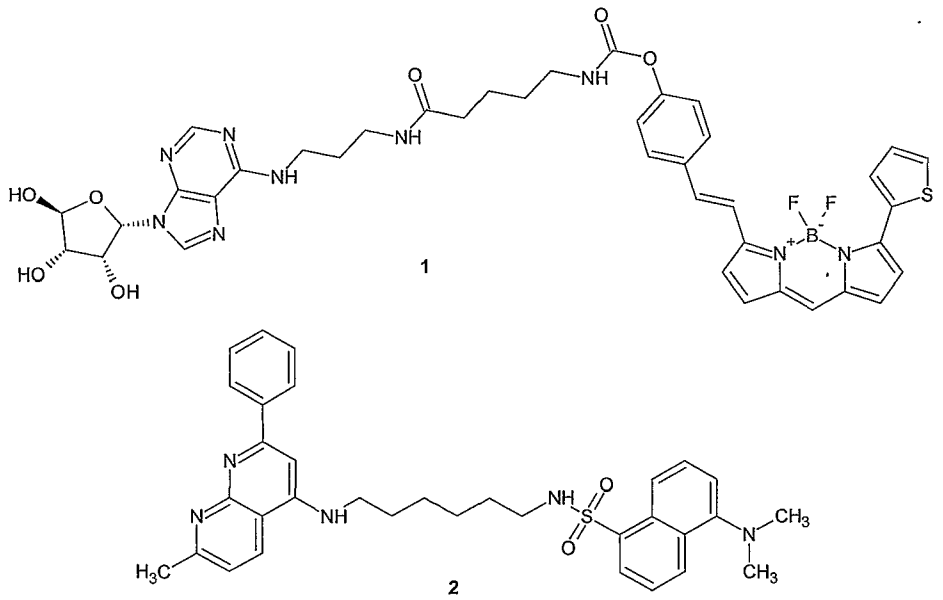


Figure 2: Fluorescent ligands for the adenosine A1-receptor (**1**,**2**)

The serotonin receptor (5-HT) has been implicated in a number of pathological disorders including; irritable bowel syndrome, gastroparesis, urinary incontinence, cardiac atrial arrhythmia and learning.[44] Recently, a large body of *in vitro* evidence has shown an intervention of these receptors in the secretion of the non-amyloidogenic soluble form of the amyloid precursor protein implicated in Alzheimer disease.[45] Fluorescent ligands for these receptors should represent valuable tools for the visualization and the study of binding and activation mechanisms of these receptors in different cell types and different tissues. Consequently, serotonin receptors constitute a valuable target for the design of new drugs and

fluorescent probes to study these receptors which may be of value in the research of neurological disorders. A recent study reported on a series of extended chain length 1-(2-methoxyphenyl)piperazine derivatives conjugated to environment-sensitive fluorescent moieties such as, 4-amino-1,8-naphthalimide, 4-dimethylaminophthalimide and dansyl, synthesised and evaluated for the 5-HT_{1A} receptor subtype.[46] The novel ligands displayed very high to moderate nanomolar affinity for the 5-HT_{1A} receptor and good fluorescence properties with high stokes shifts. Several of the newly prepared ligands displayed 5-HT_{1A} receptor and fluorescent properties suitable for use in fluorescence microscopy. In particular, the fluorescent ligand **3** (Figure 3) showed a favorable combination of 5-HT_{1A} receptor affinity (Ki = 0.67 nM) and stokes shift (excitation = 400 nm, emission = 496 nm) to be utilised using modern fluorescent imaging techniques to study serotonin receptor subtypes especially in the CNS.

The first report of fluorescent antagonists for human 5-HT_4 receptors was published by the group of Berque-Bestel *et al.*[33] These novel fluorescent ligands were synthesised based on ML10302, a potent 5-HT_4 receptor agonist. The ligands were conjugated to three fluorescent moieties, dansyl, naphthalimide, and NBD, through alkyl chains differing in length (Figure 3). The synthesised molecules were evaluated in binding assays on cloned human 5-HT_4 receptors stably expressed in C6 glial cells with [^{3}H]GR113808 as the radioligand. For ML10302-based ligands, dansyl (**4**) and NBD (**5**) derivatives attached through a chain length of one carbon atom (Figure 4), led to affinities close to the affinity of ML10302. Compound **6** containing the naphthalimide fluorophore was found to be the most potent ligand producing an inhibition of the 5-HT_4 stimulated cyclic AMP synthesis with nanomolar Ki values. Ligand **6** was further used in fluorescence microscopy experiments in order to label the 5-HT_4 receptors transfected on C6 glial cells. Through the use of fluorescent ligand **6** the subcellular localization of these receptors was more precisely determined using confocal laser scanning microscopy than what was previously possible with the use of radioligands.

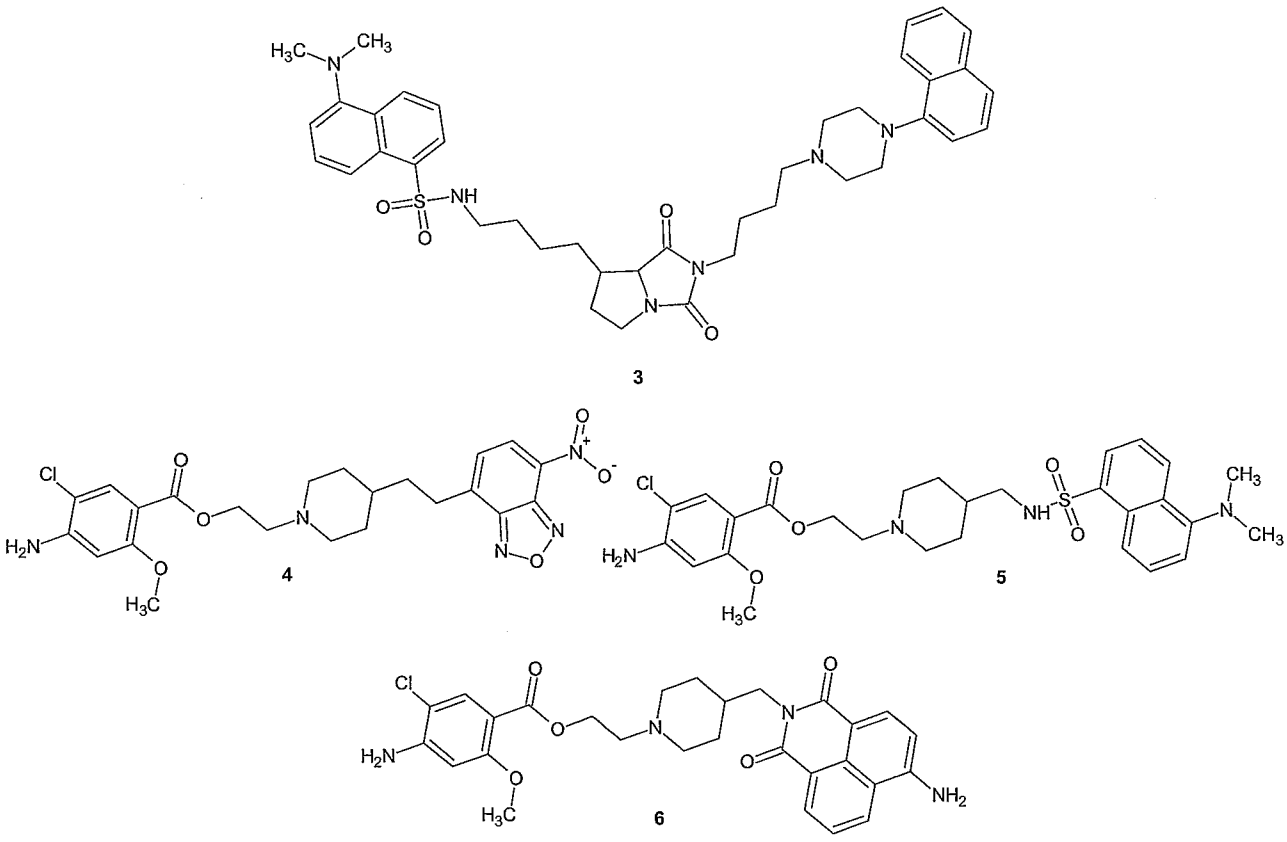
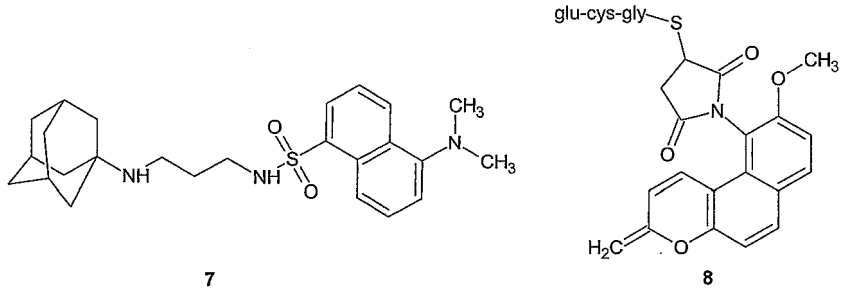


Figure 3: Fluorescent ligands for different subtype serotonin receptors (**3-6**)

The *N*-methyl-D-aspartate (NMDA) receptor has been suggested as a CNS drug target through its involvement in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease.[47] Overstimulation of the NMDA receptor by an excess of the endogenous neurotransmitter glutamate during pathological conditions leads to excessive influx of extracellular calcium into neuronal cells, resulting in cell death, a process known as excitotoxicity.[48] Currently radioligand binding techniques are used to study NMDA receptor pharmacology and physiology, especially with regard to competitive displacement assays.[3,49,50] Our group has reported the design of fluorescent ligands for the NMDA receptor to potentially develop high-throughput competition binding dispacement assays and to enable the development of novel fluorometric assays to assist in the quest for effective neuroprotective strategies.[34] A series of fluorecent ligands were synthesised through the conjugation of various fluorophores, differing in chain length, to the known NMDA receptor antagonist amantadine.[34] All the novel fluorescent ligands showed improved calcium flux modulation when compared to the lead structures' known NMDA receptor agonism. Generally it was observed that the an increase in chain length improved NMDA receptor activity which may indicate that deeper immersion into the NMDA receptor is necessary for

stronger interaction of these fluorescent ligands with the putative NMDA binding site. From this series one compound, a dansyl analogue (**7**, Figure 4) was further used as a fluorescent NMDA receptor ligand in a fluorescent displacement competition assay utilizing known NMDA receptor inhibitors to demonstrate the possible applications of this novel fluorescent ligand. This ligand could be used as a direct binding NMDA receptor fluorecent probe that can be quantified and used to directly study these receptors.

An attempt to develop various fluorescent probes to label glutathione (GSH) receptors, led to the serendipitous discovery of a fluorescent ligand that binds to, and antagonises, the NMDA receptor.[51] Fluorescent ligand **8** (Figure 4), a GSH adduct, was shown to displace the competitive NMDA antagonist [3H]-CGP 39653 with a higher affinity than known NMDA receptor inhibitors in rat synaptic membranes.[51] In rat cortical experiments **8** reversibly blocked both NMDA- and cysteine-induced depolarization. This study shows that **8** may be a useful selective NMDA antagonist which may be utilised as a fluorescent ligand to study the NMDA receptor.



7 8

Figure 4: NMDA receptor fluorescent analogues (**7**,**8**)

The discovery of nitric oxide (NO) as a biological signaling agent stimulated a range of research activities with the ultimate aim of elucidating the precise biological functions of NO.[52-54] The overproduction of NO was implicated in various neurological disorders, which includes amongst others, Parkinson's, Alzheimer's and Huntington's diseases, as well as neuronal damage due to stroke.[53] One critical mission of research is to pinpoint the location of NO formation and NO-induced events at the cellular level. Thus, an indicator capable of visualizing NO in the CNS is desired, to provide selective and direct information about its production and migration. A very promising approach for achieving this goal is the use of fluorescence methodologies.[54] One small-molecule fluorescent ligand described for NO detection was *o*-diaminonaphthalene also known as DAN, which shows an increase in fluorescent intensity in the presence of NO oxidation products (e.g., NO_2-, N_2O_3). The disadvantage of this ligand is that it can serve only as an indirect NO sensor.[54] DAN also

requires high-energy excitation for fluorescence imaging, which can damage cells. To improve upon the properties of DAN, several *o*-diaminofluorescein compounds (DAFs) have been reported as fluorescent NO indicators. These compounds still requires an oxidized NO species and like DAN, DAFs are also indirect NO sensors.[54,55]

Small molecule fluorescent ligands to directly detect NO by utilizing the chemistry of transition-metal complexes has also been reported.[56] Anthrancenyl (**8**) and dansyl (**9**,**10**) fluorescent ligands (Figure 5) were developed to form CuII complexes as candidates for detecting nitric oxide (NO) by fluorescence. A decrease in fluorescence compared to that of the free ligands was measured following the formation of the corresponding CuII complexes. The fluorescence of the compounds was restored in the presence of various concentrations of NO solutions. Spectroscopic studies revealed that the NO-induced fluorescent increase in these CuII complexes occurs by reduction to CuI. This study indicated that metal-based fluorescent ligands for NO detection in biological systems may hold advantage to directly study NO utilizing fluorescent techniques to gain a better understanding of the involvment of NO in neurological disorders.

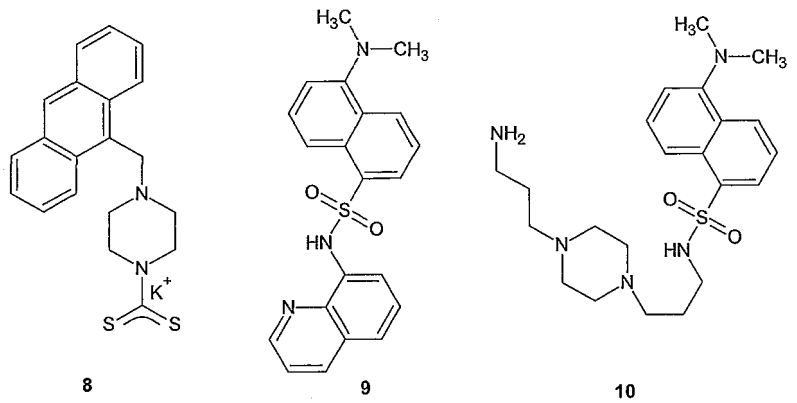


Figure 5: Novel nitric oxide fluorescent scensors (**8-10**)

Joubert *et al.*,[57] recently synthesised a series fluorescent polycyclic ligands to study the CNS nitric oxide synthase (NOS). NOS catalyzes the oxidation of *L*-arginine to *L*-citrulline in the CNS, generating NO, a critical neurotransmitter,[56] of which overproduction may lead to various neurological diseases.[53] Since neuronal NOS plays a critical role in the production of neuronal NO, it is considered to be a promising neuroprotective therapeutic target.[59] The results from inhibition studies showed that four compounds including; indazole-pentacycloundecane (**11**), coumarin-adamantane (**12**), cyanoisoindole-adamantane (**13**) and dansyl-adamantane (**14**), exhibited IC_{50} values of less than 1 μM for the NOS enzyme (Figure 6). These compounds could possibly be used as

molecular fluorescent probes in the development of high-throughput screening NOS displacement assays. To our knowledge this is the first attempt to design fluorescent ligands to directly study binding of the neuronal NOS enzyme, thus circumventing the use of traditional radioligand binding techniques routinely used in competition binding assays.[57]

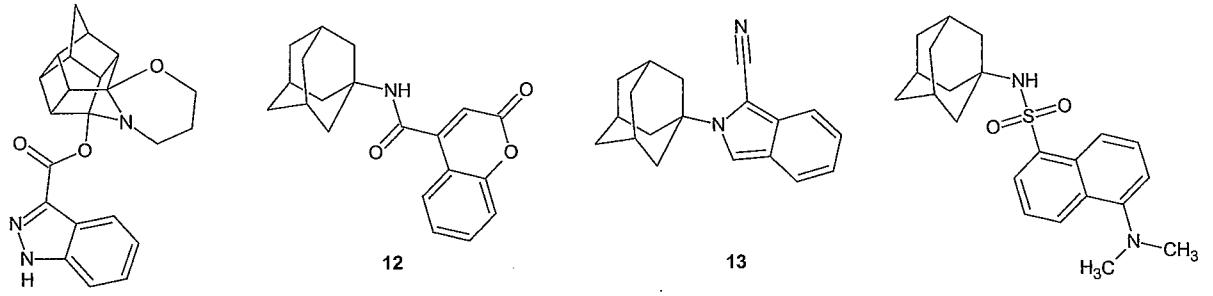
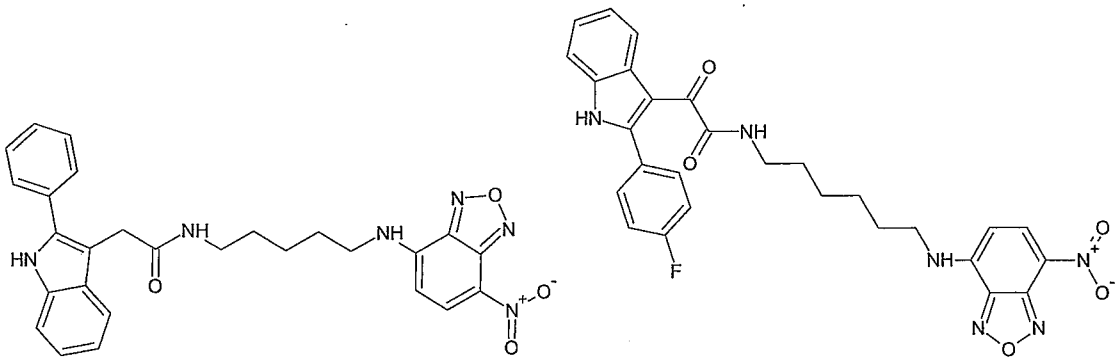


Figure 6: Fluorescent ligands (**11-14**) to study the enzyme neuronal nitric oxide synthase (NOS)

The peripheral-type benzodiazepine receptor (PBR) is an 18 KDa protein located on the outer mitochondrial membrane,[60] which is directly or indirectly implicated in numerous cellular functions.[61,62] PBR expression is also significantly enhanced in neurodegenerative diseases such as Huntington's and Alzheimer's diseases and multiple scleroses, as well as in various forms of brain injury and inflammation.[62] These findings have stimulated the development of fluorescent ligands targeting PBR as tools to image and measure the expression level of this protein in neurodegenerative disorders.

The group of Kozikowski *et al.*, reported a fluorescent high-affinity PBR ligand (**15**, Figure 7) related to the 2-aryl-3-indoleacetamide prototype PBR antagonist, FGIN-1-27, which provides a tool to allow direct imaging by fluorescence microscopy of the PBR protein in living cells.[63] This fluorescent ligand retained full ability to displace the radiolabeled isoquinoline from the isoquinoline binding site on the PBR protein and specifically labeled the intracellular localization of the PBR. This compound may be a useful tool to probe the localization and function of the PBR in different tissues associated with neurodegeneration.[63]



15 16

Figure 7: Peripheral-type benzodiazepine receptor fluorescent ligands (**15,16**)

A more recent study by Taliani *et al.*, described the synthesis of fluorescent ligands for the PBR, based on *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamides, a potent and selective class of PBR ligands previously described by the same group.[64] They selected the well-known 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) fluorophore, because its small size does not generally affect affinity of the parent ligand. Moreover, NBD-containing compounds typically exhibit a low quantum yield in an aqueous solution, but they become highly fluorescent in nonpolar solvents or when bound to membranes or to hydrophobic clefts in proteins.[64] All the new fluorescent ligands reported were found to be moderately to highly potent (*K*i values varying from the 12 – 994 nM) PBR antagonists as determined by radioligand binding competition experiments. The PBR radiolabel [^{3}H]PK 11195 was used and the assays were performed on rat kidney mitochondrial membranes.[65] Results from the binding studies showed that an increase in chain length from a short ethylene chain to chains of 4 to 6 methylene units displayed higher affinities for the PBR. The length of the spacer alkyl chain probably introduced some flexibility that favored binding of the ligands into the receptor binding site. These results again show the importance of optimal chain length in the design of fluorescent ligands. The most potent fluorescent probe (**16**; Figure 7) was selected for further fluorescent microscopy experiments aimed at labeling PBR at the mitochondrial level in rat C6 glioma cells. Under these conditions, cells were uniformly stained in the cytoplasm (Figure 8A,B) and no significant differences were observed with higher ligand concentration. Competitive binding studies were done on cells pre-incubated with different concentrations of non-fluorescent known high affinity PBR antagonists, including **PK 11195** and **Ip**.[65] It was then stained with 10 μM **16**. The fluorescent staining was significantly decreased by both the PBR antagonists at 10 μM concentration (Figure 8C,D). The specificity of compound **16** was further demonstrated by the complete displacement of **16** staining when the cells were pre-incubated with higher concentrations of **PK 11195** (Figure 8E). Compound **16** can be

considered as a fluorescent probe for investigating the PBR and to replace PBR radioligands in studies on the localization, expression level, structure and physiological/pathological roles of this receptor in the development of neurodegeneration.

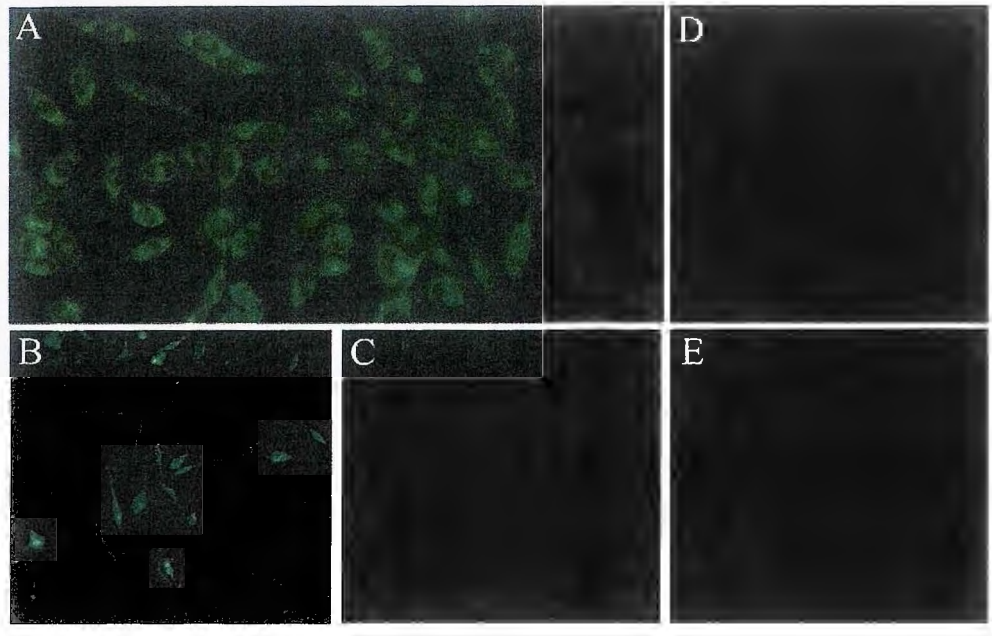
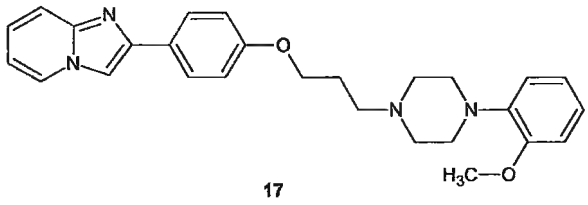


Figure 8: Rat C6 glioma cells stained with 10 μM **16** (A,B); cells stained with 10 μM **16** in the presence of 10 μM **PK 11195** (C); cells stained with 10 μM **16** in the presence of 10 μM **Ip** (D); and cells stained with 10 μM **16** in the presence of 50 μM **PK 11195** (E) (with permission from Taliani *et al*).[64]

The D_3 receptor is responsible for mediating the physiological effects of dopamine in diverse tissues such as the brain and kidney and regulates various cellular functions.[66] Initial pharmacological studies have investigated D_3 receptors as potential therapeutic targets for the treatment of Parkinson's disease because it was evident that the anti-Parkinsonian effects of dopaminergic agonists were due to activation of D_2 and/or D_3 receptors.[66] Recent *in vitro*, *in vivo*, and clinical observations have provided compelling evidence that dopamine D_3 receptors play a major role in the expression of the neuroprotective and neurorestorative actions of dopaminergic agonists.[66,67] Therefore, the recruitment of dopamine D_3 receptors would appear to be a promising strategy for the development of more effective agents for preventing the degeneration and could also be promising for the restoration of the function of dopaminergic neurons in Parkinson's disease.[67]

The group of Leipoldo *et al.*, reported the synthesis of a series of fluorescent ligands for the dopamine D_3 receptor designed on the basis of the structure of the known dopamine D_3 ligand 1-(2-methoxyphenyl)piperazine and of the reported fluorescent moiety 2-phenylimidazo[1,2-*a*]pyridine.[68] High-affinity ligands for the human D_3 receptor were

obtained with *Ki* values ranging from 0.72-385 nM, as determined using radioligand binding studies. One of the most potent D_3 ligands in this series **17** (*Ki* = 1.6 nM) showed good Stokes shift and high quantum yields. This prompted further investigation of **17** as a potential fluorescent ligand to visualize D_3 receptors expressed in CHO cells in a preliminary experiment by fluorescent microscopy. Unfortunately no fluorescent labeling by **17** was detected even at high concentrations (3-1000 nM). The authors ascribed this to cell autofluorecence and unfavorable environmental polarity. They however, concluded that two-photon microscopy might allow visualization of this probe in living cells because of the inherent advantage of nearinfrared excitation using this technique, thus avoiding excitation wavelengths typical of one-photon fluorescence microscopy (300-560 nm) that may cause damage to the substrates and cell autofluorescence.

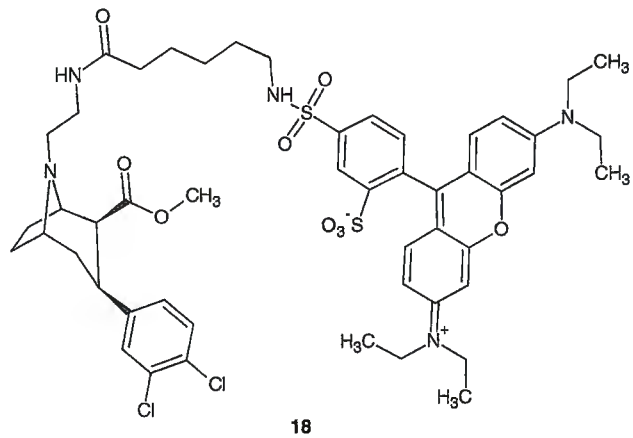


17

Figure 9: Potent fluorescent ligand for the dopamine D_3 receptor

Studies using a cocaine analogue, which contained the fluorescent moiety NBD, were conducted to explore the biophysical properties of the cocaine binding site in the rat serotonin transporter (SERT).[69] This analogue was not conducive to visualising the SERT or the homologous dopamine transporter (DAT) in living cells most likely because of the low brightness of NBD (quantum yields for NBD derivatives in water are <0.1).[70] More recently, cocaine-fluorophore conjugates have been reported that reproduce affinity constants of radiolabled [^{3}H]cocaine and thus represent potential non-radioactive bioimaging tools with particular application toward the development of therapeutic antibodies against cocaine [71]. A study by the group of Cha *et al.*,[72] synthesised a series of 2-substituted and *N*-substituted rhodamine-labeled fluorescent ligands of cocaine to identify a high-affinity probe that would enable visualization of the dopamine transporter (DAT) in living cells and lower background staining than previously prepared ligands.[70] Moreover they used fluorescent moieties with higher photostability and quantum yield than NBD, such as the rhodamine derivatives which display quantum yields of 0.95-1.00.[73] The resulting *N*-substituted rhodamine-labeled ligand (**18**; Figure 10) provided the highest DAT binding affinity in competition binding experiments using [^{3}H]CFT as the radioligand in COS-7 cells (*Ki* = 18 nM). Visualization of the DAT using **18** was demonstrated by CLSM in HEK293 cells. HEK293 cells stably expressing the

hDAT, or nontransfected controls cells, were incubated with 10 nM of **18**. DAT on the cell surface could be visualized within minutes and with increasing intensity over time, reaching maximum staining after 20 minutes (Figure 11). The possibility of using this ligand for the direct labeling of the DAT in living neuronal cells represents a new and important approach for understanding cellular targeting and trafficking in the CNS.



18

Figure 10: High affinity DAT rhodamine-labeled fluorescent ligand (**18**)

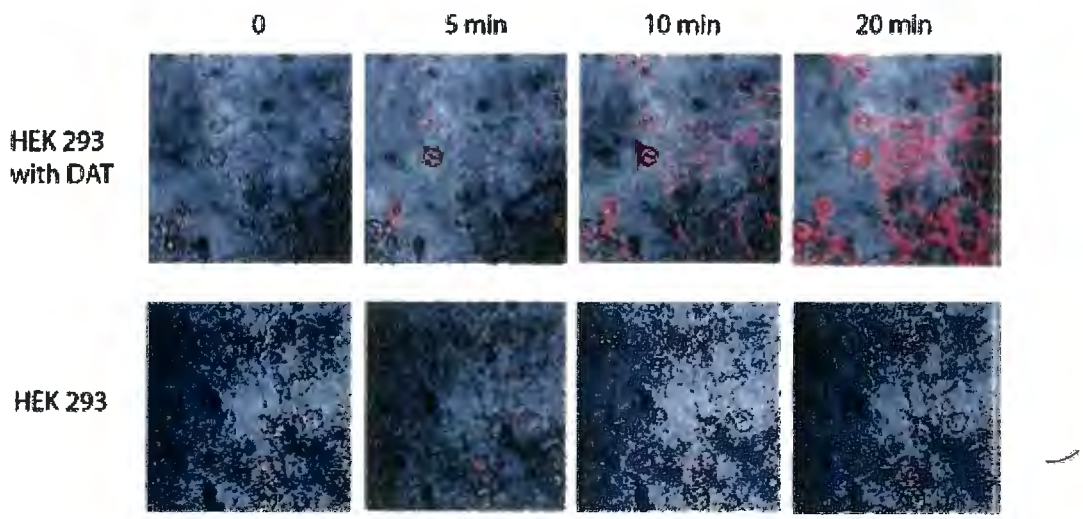


Figure 11: Visualization of DAT in HEK293 cells expressing DAT by CLSM incubated with 10 nM of fluorescent ligand **18** (with permission from Cha *et al*).[72]

The sigma receptor has recently received increasing attention as it was established that it belongs to a class separate from the opiate receptors. Furthermore, sigma receptors are now being recognized as potential drug targets in a variety of CNS disorders, including schizophrenia, depression and drug addiction,[74-78] as well as for imaging probes.[79,80] Zeng *et al.*, recently described the design of two fluorescent ligands, **19** and **20** (Figure 12), having high *in vitro* affinity and selectivity for sigma-2 over sigma-1 receptors.[81] These compounds

were able to selectively image the subcellular localization of the sigma-2 receptor in the mitochondria, endoplasmic reticulum and in lipid rafts in living cells by CLSM and multi-photon fluorescent microscopy (MPFM). The rapid internalization of the fluorescent probes observed suggested that the sigma-2 receptors receptors are internalized, in part, *via* the endocytotic pathway. The localization of sigma-2 receptors in several organelles known to have a role in both caspase-dependent and caspase-independent pathways of cell death supports the potential of the sigma-2 receptor as a drug target for CNS disorders where apoptosis leads to the development of neurodegenerative disorders.[82]

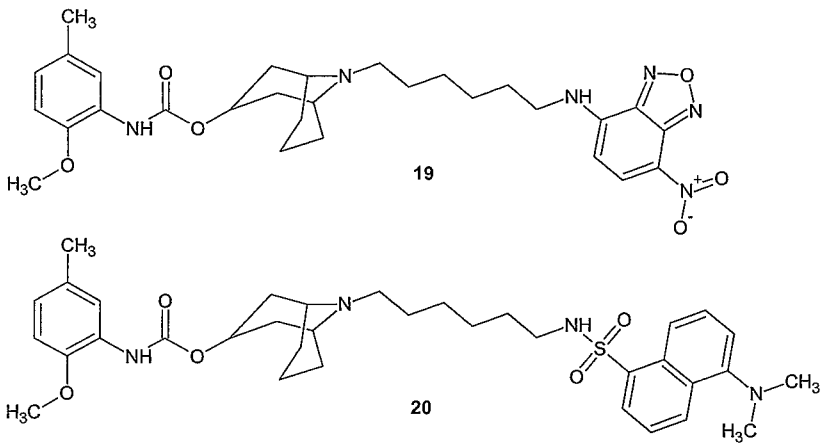


Figure 12: Sigma-2 receptor selective fluorescent ligands (**19**,**20**)

3.5. Fluorescent ligands as diagnostic tools for neurological disorders.

Fluorescent ligands have also shown potential as non-invasive diagnostic imaging tools for neurological disorders. One such disorder is Alzheimer's disease. Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that afflicts millions worldwide, is difficult to diagnose, and has no cure. It is characterized by pathological deposits of amyloid-beta peptide namely, amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) of intracellular hyperphosphorylated tau proteins.[83,84] The rational design and development of an *in vivo* functional molecular ligand that enters the brain and enables the individual detection of NFTs and Aβ plaques would benefit approaches based on noninvasive fluorescent optical imaging or positron emission tomography (PET) to allow for routine diagnostic testing and a more precise understanding of the pathophysiology of neurodegenerative diseases.[85] For this purpose, several small molecular ligands have been developed, but none of them has been able to discriminate between NFTs and Aβ plaques with high specificity.[86] One recent study by Ojida *et al.*, described the development of a new small molecule fluorescent binuclear Zn(II) complex (**21**, Figure 13) with the first account of selective detection of NFTs over Aβ

plaques. The ligand incorporated a fluorescent BODIPY unit and two Zn(II)-2,2′-dipicolylamine complexes (**21**) as a binding site for phosphorylated amino acid residues.[86] Histological imaging using CLSM of hippocampus tissue from an AD patient revealed that the ligand fluorescently visualised deposits of NFTs with very high specificity and selectivity. This fluorescent ligand is an excellent example of a tool for the precise pathological understanding and diagnosis of tau protein-related neurodegenerative disorders utilizing fluorescent techniques.

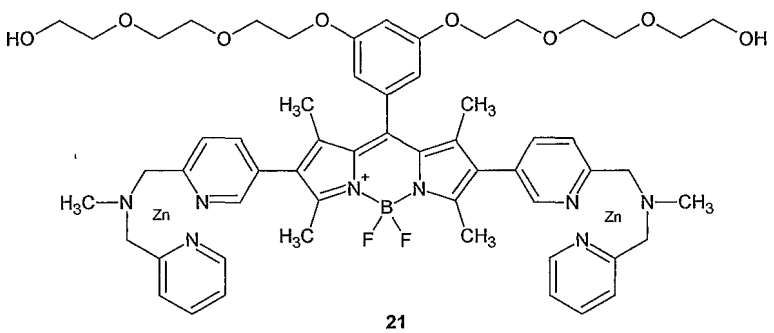


Figure 13: Small molecule fluorescent binuclear Zn(II) complex (**21**), a selective detector of neurofibrillary tangles in Alzheimer's disease

To date, a number of groups have worked on MRI[87,88] and PET[89,90] ligands for amyloid plaques. Notably, the PET ligand Pittsburgh Compound B (PIB) has shown promise in early clinical trials and is currently used in a number of human studies.[91] A recent development in molecular imaging for Aβ plaques in AD is near-infrared fluorescent (NIFR) ligands that emit characteristic fluorescence signal only when bound to Aβ plaques which may translate into the discovery of novel clinical diagnostic tools for prodromal diagnosis of this debilitating neurodegenerative disorder.[85,92,93] NIRF ligands are typically small molecule fluorescent dyes that possess suitable properties to be imaged deeper in living tissues. NIRF ligands are designed to absorb and emit light in the near-infrared region (650-950 nm), at this end of the spectrum there is much less scattering and absorption of light rays with water and other tissue components thus reducing background and autofluorescence and, therefore, improving sensitivity of detection.[85,94] The simple synthesis, low-cost, and long shelf-life of NIRF ligands, together with the low cost of optical imaging devices, present an attractive alternative to MRI and PET techniques.[85] Two such probes, compounds **22** and **23** (Figure 14) show absorption and emission spectra that are shifted to the near infrared region once bound to Aβ.[85,88] These probes exhibited Aβ-dependent changes in fluorescence quantum yield, lifetime, and emission spectra that may be imaged microscopically or *in vivo* using new lifetime and spectral fluorescence imaging techniques. NIRF probes that turn on when bound

to Aβ will improve amyloid detection and may enable quantitative molecular imaging *in vivo*. Some of the major hurdles in the development for a succesful NIRF amyloid optical marker are to develop probes that emit above 700 nm, bind amyloid plaques, show good plaque to background contrast and washout kinetics, and cross the blood-brain barrier after intravenous injection. A fluorophore that emits between 700 and 850 nm is optimal because tissue absorption at these wavelengths is the lowest, allowing deeper light penetration.[85-88] To date no NIRF amyloid imaging agent has been developed with these optimal properties.

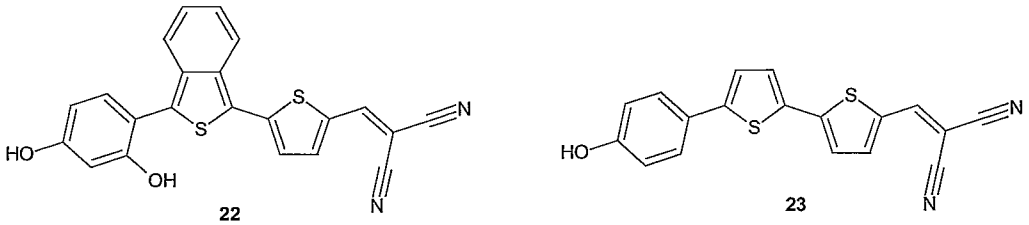


Figure 14: Near-infrared fluorescent ligands as imaging probes (**22**,**23**) for amyloid-beta (Aβ) plaques in Alzheimer's disease

Bai *et al.* described the first NIRF probe that specifically binds cannabinoid CB2 receptors and has potential use in *in vivo* imaging. CB2 receptor is an attractive target for the noninvasive imaging of neuroinflammation because its expression is induced in resident microglial cells after cerebral ischemia, injury and in neuroinflammatory diseases.[95]

Another example of potentially useful diagnostic fluorescent agents was described by Hadrich *et al.*[96] Their group synthesised a series of fluorescent ligands with affinity for the norephinephrine transporter (NET), which is overexpressed in neural crest tumors such as neuroblastomas. The fluorescent ligands were conjugated to known selective agents based on the radiolabeled m-iodobenzylguanidine tumor-seeking radioactive drug which is transported into the tumor cells by the neuronal NET and expressed in almost all neuroblastoma cells. Two compounds which incorporated the dansyl (**24**) and NBD (**25**) fluorescent groups showed high affinity for the NET (IC_{50} < 50 nM; Figure 14). More recently a fluorescent stilbazolium dye (**26**) was developed which also showed high selectivity for NET as evaluated in HEK-293 cells. These probes have potential as diagnostic agents for labeling neuroblastoma tumor cells and as a substrate suitable for imaging and reporting of transporter function of NET, both *in vitro* and *in vivo*.

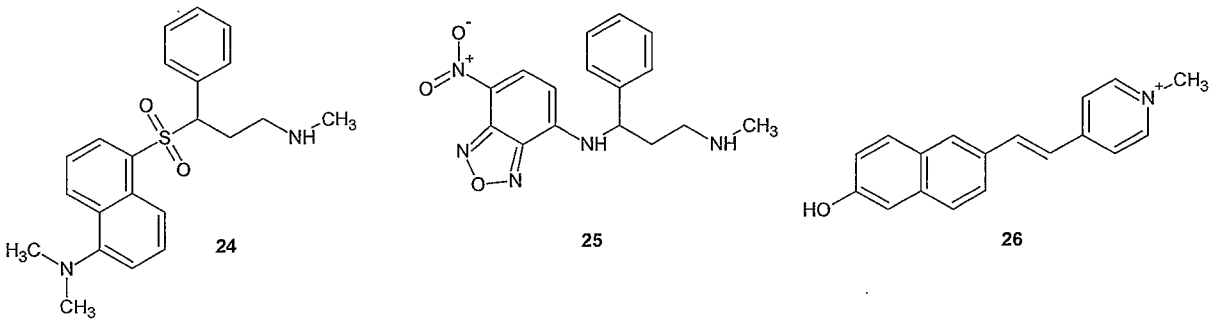


Figure 15: Fluorescent ligands **(24-26)** with high affinity for the norephinephrine transporter

Further advancement in the diagnosis for labelling tumour cells was recently described for detection of gliomas during surgery. Gliomas are malignant brain tumours that arise from glial cells of the brain which use albumin for cellular growth and are often resistant to chemotherapy.[97] These tumours grow fine extensions that infiltrate normal brain tissue and, in addition, individual tumour cells can form satellites in surrounding tissue. Therefore, it is almost impossible to remove the tumour tissue completely by surgery.[98,99] The group of Kremer *et al*, developed a fluorescent probe for fluorescence-guided surgery of malignant brain tumours using the fluorescent ligand 5-aminofluorescein covalently linked to human serum albumin to assess its efficacy and tolerability within a clinical setting to fluorescently stain the tumour tissue.[99] Fluorescence guidance using a 488-nm argon laser allowed fluorescent imaging of tumour and fine extensions thereof. It also demonstrated that albumin is a suitable carrier system for selective targeting of aminofluorescein into malignant gliomas for removing this tumour. Further studies are currently underway to evaluate the true potential of this novel development for the use of fluorescent ligands.

3.6. Conclusion

Fluorescent ligands in combination with fluorescence spectroscopy are becoming a valuable addition to the array of techniques available for scrutinizing ligand-protein interactions in biological systems. In particular, confocal laser scanning microscopy, flow cytometry and multi photon microscopy, allow for noninvasive imaging and quantification of interactions between these small molecule fluorescent ligands and target-proteins in single living cells. As with other imaging technologies, research on small molecule fluorescent ligands in combination with new sophisticated fluorescent techniques should continue to be rapid, and will provide tools to study the processes underlying many biological phenomena in the CNS. Although numerous fluorescent ligands have been reported so far, none of them are ideal for all applications. Next-generation probes should possess greater sensitivity, selectivity,

photostability and brightness, more controlled intracellular localization, and superior ADMET properties. Hybridization of fluorescent probes with other imaging agents, such as those for MRI and nuclear imaging, has also emerged and is a promising field. The future challenge lies in the consequent combination of fluorescence detection techniques with procedures to handle, manipulate and analyse molecules, molecular assemblies and biological cells at the micro- and nanometer level. The study of CNS target ligand interactions have a long way to go. Fortunately, technology is advancing rapidly and the combination of new fluorescent ligands, advances in synthetic chemistry methods and new fluorescent imaging techniques will provide us with powerful tools to study CNS biological systems in the future.

References

[1] M. Auer, K.J. Moore, F.J. Myers-Almes, R. Guenther, A.J. Pope, K.A. Stoekli, *Drug Disc Today* 1998, 3, 457-465.

[2] J.C. McGrath, S. Arribas, C.J. Daly, *Trends Pharm Sci* 1996, 17, 393-399.

[3] C.J. Daly, J.C. McGrath, *Pharm Therap* 2003, 100, 101-118.

[4] R.T. McCabe, A.H. Newman, P. Skolnick, *J Pharm Exp Terap* 1992, 262(2), 734–740.

[5] S. H. Snyder, *J Am Med Assoc,* 1989, 261, 3126-3129.

[6] R.W.Olsen, R.T. McCabe, J.K. Wamsley, *J Chem Neuroanat* 1990, 3, 59–76.

[7] J.D. Wulfkuhle, K.H. Edminston, L.A. Liotta, E.F. Petricoin, *Nat Clin Pract Onc* 2006, 3, 256-268.

[8] L.L. Leung, *J Clin Invest* 1984, 74(5), 1764–1772.

[9] N. Baindur, D.J. Triggle, *Drug Dev Res* 1994, 33, 373-398.

[10] N. Baindur, D.J. Triggle, *Med Res Rev* 1994, 14, 591-664.

[11] M. Leopoldo, E. Lacivita, F. Berardi, R. Perrone, *Drug Discov Today* 2009, 14, 709-712.

[12] A.P. Tairi, R. Hovius, H. Pick, H. Blasey, A. Bernard, A. Surprenant, K. Lunstrom, H. Vogel, *Biochem* 1998, 37, 15850-15864.

[13] B.W. Madsen, C.L. Beglan, C.E. Spivak, *J. Neurosci. Methods* 2000, 97, 123-131.

[14] A. Beaudet, D. Nouel, T. Stroh, F. Vandenbulcke, C. Dal-Farra, J.P. Vincent, *Braz. J Med Biol Res* 1998, 31, 1479-1489.

[15] S. Terrillon, L.L. Cheng, S. Stoev, B. Mouillac, C. Barberis, M. Manning, T. Durroux, *J Med Chem* 2002, 45, 2579-2588.

[16] G. Turcatti, S. Zoffmann, J.A. Lowe, S.E. Drozda, G. Chassaing, T.W. Schwartz, A. Chollet, *J Biol Chem* 1997, 272, 21167-21175.

[17] G. Turcatti, K. Nemeth, M.D. Edgerton, U. Meseth, F. Talabot, M. Peitsch, J. Knowles, H. Vogel, A. Chollet, *J Biol Chem* 1996, 271, 19991-19998.

[18] M. Macchia, F. Salvetti, S. Bertini, V. Di Bussolo, L. Gattuso, M. Gesi, M. Hamdan, N.K. Klotz, T. Laragione, A. Lucacchini, F. Minutolo, S. Nencetti, C. Papi, D. Tuscano, C. Martini, *Bioorg Med Chem Lett* 2001, 11, 3023-3026.

[19] D. Hadrich, F. Berthold, E. Steckhan, H. Bonisch, *J Med Chem* 1999, 42, 3101-3108.

[20] H. Chen, N.N. Chung, C. Lemieux, B. Zelent, J.M. Vanderkooi, I. Gryczynski, B.C. Wilkes, P.W. Schiller, *Biopolymers* 2005, 80, 325-331.

[21] M.E. Vazquez, J.B. Blanco, S. Salvadori, C. Trapella, R. Argazzi, S.D. Bryant, Y. Jinsmaa, L.H. Lazarus, L. Negri, E. Giannini, R. Lattanzi, M. Colucci, G. Balboni, *J Med Chem* 2006, 49, 3653-3658.

[22] M.D. Petersen, S.V. Boye, E.H. Nielsen, J. Willumsen, S. Sinning, O. Wiborg M. Bols, *Bioorg Med Chem* 2007, 15, 4159-4174.

[23] J.G. Haugland, J. Gregory, M.T.Z. Spence, I. Johnson, E. Miller, *Handbook of fluorescent probes and research products*. USA: Molecular probes. 2002.

[24] S.B. Brown, *An Introduction to Spectroscopy for Biochemists*; Academic Press: London, 1980.

[25] I.D. Campbell, R.A. Dwek, *Biological Spectroscopy*; The Benjamin/Cummings Publishing Co. Inc., 1984.

[26] I. Song, E.J. Hennink, I.T. Young, H.J. Tanke, *Biophys J* 1995, 68, 2588-2600.

[27] I. Song, J.W. Varma, J.W. Verhoeven, H.J. Tanke, *Biophys J* 1996, 70, 2959-2968.

[28] B. Herman, *Fluorescence Microscopy*; 2nd ed.; BIOS Scientific Publishers: Oxford, UK, 1998.

[29] A.S. Yates, S.W. Doughty, D.A. Kendall, B. Kellam, *Bioorg Med Chem* 2005, 15, 3758–3762.

[30] G. Balboni, S. Salvadori, A. Dal Piaz, F. Bortolotti, R. Argazzi, L. Negri, R. Lattanzi, S.D. Bryant, Y. Jinsmaa, L.H. Lazarus, *J Med Chem* 2004, 47, 6541–6546.

[31] J.H. Cha, M. Zou, E.M. Adkins, S.G.F. Rasmussen, C.J. Loland, B. Schoenenberger, U. Gether, A.H. Newman, *J Med Chem* 2005, 48, 7513-7516.

[32] R.J. Middleton, S.J. Briddon, Y. Cordeaux, A.S. Yates, C.L. Dale, M.W. George, J.G. Baker, S.J. Hill, B. Kellam, *J Med Chem* 2007, 50, 782–793.

[33] I. Berque-Bestel, J.L. Soulier, M. Giner, L. Rivail, M. Langlois, S. Sicsic, *J Med Chem* 2003, 46, 2606-2620.

[34] J. Joubert, S. van Dyk, I. R. Green, S. F. Malan. *Eur J Med Chem* 2011, 46, 5010-5020.

[35] M.E. Va´zquez, J.B. Blanco, S. Salvadori, C. Trapella, R. Argazzi, S.D. Bryant, Y. Jinsmaa, L.H. Lazarus, L. Negri, E. Giannini, R. Lattanzi, M. Colucci, G. Balboni, *J Med Chem* 2006, 49, 3653–3658.

[36] F. Krieger, A. Mourot, R. Araoz, F. Kotzyba-Hibert, J. Molgó, E. Bamberg, M. Goeldner, *Chembiochem* 2008, 9, 1146–11146.

[37] J.M. Tam, R. Upadhyay, M.J. Pittet, R. Weissleder, U. Mahmood, *Mol Imaging* 2007, 6(4), 269–276.

[38] Y. Wang, C.A. Mathis, G.F. Huang, M.L. Debnath, D.P. Holt, L. Shao, W.E. Klunk, *J Mol Neurosci* 2003, 20(3), 255–260.

[39] R.J. Middleton, B. Kellam, *Curr Opin Chem Biol* 2005, 9, 517–525.

[40] B. Rademaker, K. Kramer, A. Bast, H. Timmerman, *Res Commun Chem Pathol Pharmacol* 1988, 60, 147-159.

[41] V.R. Ralevic, G. Burnstock, *Pharmacol Rev* 1998, 50, 413-492.

[42] C.V. Gomes, M.P. Kaster, A.R. Tomé, P.M. Agostinho, R.A. Cunha. *Biochimica et Biophysica Acta* 2011, 1808, 1380–1399.

[43] M. Macchia, S.Bertini, V.Di Bussolo, C. Manera, C. Martini, F. Minutolo, C. Mori, G. Saccomanni, D. Tuscano, P.L. Ferrarini, *Il Farmaco* 2002, 57(10), 783-786.

[44] M. Langlois, R. Fischmeister, *J Med Chem* 2003, 46, 1-26.

[45] S.J. Robert, J.L. Zugara, R. Fischmeister, A.M. Gardier, F. Lezoualc'h, *J Biol Chem* 2001, 276, 44881-44888.

[46] D. Alonso, H. Vazquez-Villa, A.M. Gamo, M.F. Martínez-Esperon, M. Tortosa, A. Viso, R.F. de la Pradilla, E. Junquera, E. Aicart, M. Martín-Fontecha, B. Benhamu, M.L. Lopez-Rodríguez, S. Ortega-Gutierrez, *Med Chem Lett* 2010, 1, 249–253.

[47] J.A. Kemp, R.M. McKernan, *Nat Neurosci* 2002, 5, 1039-1042.

[48] M.F. Cano-Abad, M. Villarroya, A.G. García, N.H. Gabilan, G. Manuela, M.G. López, *J Biol Chem* 2001, 276, 39695-39704.

[49] R.T. McCabe, D.R. Mahan, R.B. Smith, J.K. Wamsley, *Pharmacol Biochem Behav* 1990, 37, 365-370.

[50] W.J. Geldenhuys, S.F. Malan, T. Murugensan, C.J. Van Der Schyf, *Bioorg Med Chem* 2004, 12, 1799-1806.

[51] C.A. Shaw, B.A. Pasqualotto, K. Curry, S.U. Kim, K.A. LeCompte, M.E. Langmuir, *J Neurosci Meth* 1999, 93, 21–26.

[52] D.D. Thomas, L.A. Ridnour, J.S. Isenberg, W. Flores-Santana, C.H. Switzer, S. Donzelli S, P. Hussain, C. Vecoli, N. Paolocci, S. Ambs, C.A. Colton, C.C. Harris, D.D. Roberts, D.A. Wink, *Free Radical Biol Med* 2008, 45(1), 18–31.

[53] S. Moncada, R.M. Palmer, E.A. Higgs, *Pharmacol Rev* 1991, 43(2), 109–142.

[54] M.H. Lim S.J. Lippard, *Inorg Chem* 2006, 45, 8980-8989.

[55] S.A. Hilderbrand, M.H. Lim, S.J. Lippard, *Topics in Fluorescence Spectroscopy*; C.D. Geddes, J.R. Lakowicz, Eds.; Springer: New York, 2005; pp 163-188.

[56] T. Nagano, T. Yoshimura, *Chem Rev* 2002, 102, 1235-1270.

[57] J. Joubert, S. van Dyk, S.F. Malan, *Bioorg Med Chem* 2008, 16, 8952-8958.

[58] A.A. Larson, K.J. Kovacs, J.C. Cooper, K.F. Kitto, *Pain* 2000, 86, 103-111.

[59] G.J. Southan, C. Szabó, *Biochem Pharmacol* 1996, 51(4), 383–394.

[60] M.L. James, S. Selleri, M. Kassiou, *Curr Med Chem* 2006, *13*, 1991-2001.

[61] P. Casellas, S. Galiegue, A.S. Basile, *Neurochem In* 2002, *40*, 475-486.

[62] V. Papadopoulos, L. Lecanu, R.C. Brown, Z. Han, Z.X. Yao, *Neurosci* 2006, 138, 749-756.

[63] A.P. Kozikowski, M. Kotoula, D. Ma, N. Boujrad, W. Tuckmantel, V. Papadopoulos, *J Med Chem* 1997, 40(16), 2435-2439.

[64] S. Taliani, F. Simorini, V. Sergianni, C. La Motta, F. Da Settimo, B. Cosimelli, E. Abignente, G. Greco, E. Novellino, L. Rossi, V. Gremigni, F. Spinetti, B. Chelli, C. Martini, *J Med Chem* 2007, 50, 404-407.

[65] G. Primofiore, F. Da Settimo, S. Taliani, F. Simorini, M.P. Patrizi, E. Novellino, G. Greco, E. Abignente, B. Costa, B. Chelli, C. Martini, *J Med Chem* 2004, 47, 1852-1855.

[66] J.N. Joyce, *Pharmacol Ther* 2001, 90, 231-259.

[67] J.N. Joyce, M.J. Millan, *Curr Opin Pharmacol,* 2007, 7, 100-105.

[68] M. Leopoldo, E. Lacivita, E. Passafiume, M. Contino, N.A. Colabufo, F. Berardi, R. Perrone, *J Med Chem* 2007, 50(20), 5043-5047.

[69] S.G.F. Rasmussen, F.I. Carroll, M.J. Maresch, A.D. Jensen, C.G. Tate, U. Gether, *J Biol Chem* 2001, *276*, 4717-4723.

[70] D. Lancet, I. Pecht, *Biochem* 1977, 16, 5150-5157.

[71] M.M. Meijler, G.F. Kaufmann, L. Qi, J.M. Mee, A.R. Coyle, J.A. Moss, P. Wirsching, M. Matsushita, K.D. Janda, *J Am Chem Soc* 2005, 127, 2477-2484.

[72] J.H. Cha, M. Zou, E.M. Adkins, S.G.F. Rasmussen, C.J.L.B. Schoenenberger, U. Gether, A.H. Newman *J Med Chem* 2005, 48, 7513-7516.

[73] J.R. Lakovicz, Principles of Fluorescence Spectroscopy; Kluwer Academic/Plenum Publishers: New York, 1999.

[74] J. E. Bermack, G. Debonnel, *J Pharmacol Sci* 2005, 97, 317-336.

[75] J. A. Fishback, M. J. Robson, Y. T. Xu, R. R. Matsumoto, *Pharmacol Ther* 2010,127, 271–282

[76] J. Wang, A. L. Mack, A. Coop, R. R. Matsumoto, *Eur Neuropsychopharmacol* 2007, 17, 708-716.

[77] E.C. Nguyen, K.A. McCracken, Y. Liu, B. Pouw, R.R. Matsumoto, *Neuropharmacology* 2005, 49, 638-645.

[78] R.R. Matsumoto, Y. Liu, M. Lerner, E.W. Howard, D. J. Brackett, *Eur J Pharmacol* 2003, 469, 1-12.

[79] T. L. Collier, R. N. Waterhouse, M. Kassiou, *Curr Pharm* 2007, 13, 51-72.

[80] M. Kassiou, R. F. Dannals, X. Liu, D. F. Wong, H. T. Ravert, U. A. Scheffel, *Bioorg Med Chem* 2005, 13, 3623-3626.

[81] C. Zeng, S. Vangveravong, J. Xu, K.C. Chang, R.S. Hotchkiss, K.T. Wheeler, D. Shen, Z.P. Zhuang, H.F. Kung, R.H. Mach, *Cancer Res* 2007, 67, 6708–6716.

[82] S. Vangveravong, J. Xu, C. Zeng R.H. Mach. *Bioorg Med Chem* 2006, 14, 6988–6997.

[83] B.T. Hyman, J.Q. Trojanowski, *J Neuropathol Exp Neurol* 1997, 56(10), 1095–1097.

[84] M. Goedert, M.G. Spillantini, *Science* 2001, 314, 777–781.

[85] S.B. Raymond, J.S. I.D. Hills, E.E. Nesterov, M. Timothy, M. Swager B.J. Bacskai. *Eur J Nucl Med Mol Imaging* 2008, 35, S93–S98.

[86] A. Ojida, T. Sakamoto, M. Inoue, S. Fujishima, G. Lippens, I. Hamachi. *J Am Chem Soc* 2009, 131(18), 6543-6548.

[87] J.F. Poduslo, T.M. Wengenack, G.L. Curran, T. Wisniewski, E.M. Sigurdsson, S.I. Macura, B.J. Borowski, C.R. Jack, *Neurobiol Dis* 2002, 11(2), 315–29.

[88] M. Higuchi, N. Iwata, Y. Matsuba, K. Sato, K. Sasamoto, T.C. Saido, *Nat Neurosci* 2005, 8(4), 527–533.

[89] K. Shoghi-Jadid, G.W. Small, E.D. Agdeppa, V. Kepe, L.M. Ercoli, P. Siddarth, S. Read, N. Satyamurthy, A. Petric, S.C. Huang, J.R. Barrio, *Am J Geriatr Psychiatry* 2002, 10(1), 24–35.

[90] M. Ono, A. Wilson, J. Nobrega, D. Westaway, P. Verhoeff, Z.P. Zhuang, M.P. Kung, H.F. Kung, *Nucl Med Biol* 2003, 30(6), 565–571.

[91] W.E. Klunk, H. Engler, A. Nordberg, Y. Wang, G. Blomqvist, D.P. Holt, M. Bergström, I. Savitcheva, G.F. Huang, S. Estrada, B. Ausén, M.L. Debnath, J. Barletta, J.C. Price, J. Sandell, B.J. Lopresti, A. Wall, P. Koivisto, G. Antoni, C.A. Mathis, B. Långström, *Ann Neurol* 2004, 55(3), 306–319.

[92] M. Hintersteiner, A. Enz, P. Frey, A.L. Jaton, W. Kinzy, R. Kneuer, U. Neumann, M. Rudin, M. Staufenbiel, M. Stoeckli, K.H. Wiederhold, H.U. Gremlich, *Nat Biotechnol* 2005, 23(5), 577–583.

[93] E.E. Nesterov, J. Skoch, B.T. Hyman, W.E. Klunk, B.J. Bacskai, T.M. Swager, *Angew Chem Int Ed Engl* 2005, 44(34), 5452–5456.

[94] A. Becker, C. Hessenius, K. Licha, B. Ebert, U. Sukowski, W. Semmler, B. Wiedenmann, C. GrItzinger, *Nat Biotechnol* 2001, 19, 327–331.

[95] M. Bai, M. Sexton, N. Stella, D.J. Bornhop, *Bioconjug Chem* 2008, 19, 988–992.

[96] D. Hadrich, F. Berthold, E. Steckhan, H. Bonisch, *J Med Chem* 1999, *42,* 3101-3108.

[97] A.N. Mamelak, D.B. Jacoby, *Expert Opin Drug Deliv* 2007, 4(2), 175–186.

[98] "Fluorescent cancer cells to guide brain surgeons." PHYSorg.com. 3 Apr 2009. www.physorg.com/news157976452.html

[99] P. Kremer, M. Fardanesh, R. Ding, M. Pritsch, S. Zoubaa E. Frei, *Neurosurgery* 2009, DOI:10.1227/01.NEU.0000335787.17029.67.