

***Antagonism by Selected Classical
Irreversible Competitive Antagonists:
An Investigation into the Proposed Non-
specific Mechanisms Involved***

Johannes Bodenstein

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Abstract

Title: *Antagonism by selected classical irreversible competitive antagonists: An investigation into the proposed non-specific mechanisms involved*

Many irreversible antagonists are known to bind irreversibly to pharmacological receptors. However, few studies suggest that these irreversible antagonists may also display irreversible non-specific antagonism by binding irreversibly to non-synaptic binding sites on the receptor macromolecule, whereby they modulate the signal transduction of these receptors or reduce the agonist binding affinity.

The aim of this study was to investigate whether the classical irreversible antagonists phenoxybenzamine, benextramine and 4-DAMP mustard display irreversible non-specific antagonism at various G protein-coupled receptor (GPCR) types. In addition, the subcellular mechanism whereby benextramine displays irreversible non-specific antagonism was investigated.

Three cell lines were employed to investigate the antagonism by these irreversible antagonists: Chinese hamster ovary (CHO-K1) cells transfected to express the porcine α_{2A} -adrenoceptor (α_{2A} -AR) at higher (α_{2A} -H) or lower (α_{2A} -L) numbers, human neuroblastoma (SH-SY5Y) cells that endogenously express muscarinic acetylcholine receptors (mACh-Rs), and SH-SY5Y cells transfected (5HT_{2A}-SH-SY5Y) to express the human 5HT_{2A}-serotonin receptor (5HT_{2A}-R). Cells of the appropriate cell line were pre-treated at the appropriate concentrations and incubation times with an appropriate irreversible antagonist, with or without an appropriate reversible competitive antagonist at a sufficient concentration to protect the specific receptors. This was followed by washing procedures with drug-free media to rinse any unbound or reversibly bound drugs from the cells. When appropriate, cell membranes were prepared. Receptor function was evaluated by measuring whole-cell [³H]-cAMP or [³H]-IP_x accumulation, or the binding of [³⁵S]-GTP γ S to membranes. Receptor concentrations were determined from radioligand-binding assays. In addition, the constitutive [³⁵S]-GTP γ S

binding to G_o protein before and after pre-treatment with benextramine was investigated.

Results suggest that phenoxybenzamine (100 μ M, 20 minutes) and benextramine (10 μ M, 20 minutes) display irreversible non-specific antagonism at α_{2A} -ARs when measuring G_i -mediated effects in α_{2A} -L cells, but the affinity for α_{2A} -ARs in α_{2A} -H cells was not changed. In addition, it was found that the observed irreversible non-specific antagonism by benextramine appears to be time- and concentration-dependent. When the mechanism of irreversible antagonism by benextramine was further investigated, benextramine reduced the binding of [^{35} S]-GTP γ S to α_{2A} -H membranes with protected α_{2A} -ARs, but did not modulate the constitutive binding of [^{35} S]-GTP γ S to G_o . In addition, benextramine displays irreversible non-specific antagonism by inhibiting the G_s -mediated effects of α_{2A} -ARs in α_{2A} -H cells and the G_q -mediated effects of mACh-Rs or 5HT $_{2A}$ -Rs in SH-SY5Y or 5HT $_{2A}$ -SH-SY5Y cells respectively. 4-DAMP mustard (100 nM, 20 minutes) did not display irreversible non-specific antagonism at mACh-Rs in SH-SY5Y cells, but irreversible non-specific antagonism was observed when the incubation time was increased (100 nM, 60 minutes).

In conclusion it was found that phenoxybenzamine, benextramine and 4-DAMP mustard display irreversible non-specific antagonism at typical experimental conditions. These findings confirm concerns in literature and supports the possibility that more irreversible antagonists could display irreversible non-specific antagonism, and that could influence the interpretation of data obtained with such drugs. In addition, benextramine may prove to be a useful experimental drug in studying GPCR signalling.

Keywords: *4-DAMP mustard; 5HT $_{2A}$ -serotonin receptor; α_{2A} -adrenoceptor; benextramine; G protein-coupled receptor; irreversible antagonist; muscarinic acetylcholine receptor; non-synaptic binding site; phenoxybenzamine; specific receptor.*

Opsomming

Titel: *Antagonisme deur geselekteerde klassieke onomkeerbare kompeterende antagonist: 'n Ondersoek na die voorgestelde non-spesifieke meganismes betrokke*

Talle onomkeerbare antagonist is bekend daarvoor dat hulle onomkeerbaar aan farmakologiese reseptore bind. 'n Paar studies suggereer egter dat hierdie onomkeerbare antagonist ook onomkeerbare non-spesifieke antagonisme mag openbaar deur onomkeerbaar aan non-sintopiese bindingsetels van die reseptormakromolekule te bind, en daardeur die seingeleiding van hierdie reseptore moduleer of agonisbindingsaffiniteit verlaag.

Die doel van hierdie studie was om ondersoek in te stel of die klassieke onomkeerbare antagonist fenoksibensamien, benekstramien en 4-DAMP mosterd onomkeerbare non-spesifieke antagonisme by verskeie tipes G-proteïengekoppelde reseptore openbaar. Daarby is die subcellulêre meganisme waardeur benekstramien onomkeerbare non-spesifieke antagonisme openbaar, ondersoek.

Drie sellyne is gebruik om die antagonisme deur hierdie onomkeerbare antagonist te ondersoek: Ovariumselle van die Chinese hamster (CHO-K1) getransfekteer om die α_{2A} -adrenoseptor (α_{2A} -AR) van die vark in hoër (α_{2A} -H) of laer (α_{2A} -L) hoeveelhede uit te druk, menslike neuroblastoomselle (SH-SY5Y) wat endogene muskariniese asetielcholinreseptore (mACh-R'e) uitdruk, en SH-SY5Y-selle getransfekteer (5HT_{2A}-SH-SY5Y) om die menslike 5HT_{2A}-serotonienreseptor (5HT_{2A}-R) uit te druk. Selle van die toepaslike sellyn is vooraf teen die toepaslike konsentrasies en inkubasietye met 'n toepaslike onomkeerbare antagonist behandel, met of sonder 'n toepaslike omkeerbare kompeterende antagonist in 'n voldoende konsentrasie om spesifieke reseptore te beskerm. Dit is opgevolg deur wasprosedures met geneesmiddelvrye media om enige ongebonde of omkeerbaar-gebonde geneesmiddels van die selle te spoel. Selmembrane is waar toepaslik berei. Reseptorfunksie is geëvalueer deur die akkumulasie van [³H]-cAMP of [³H]-IP_x in heelselle of die binding van [³⁵S]-GTP γ S aan membrane te meet.

Reseptorkonsentrasies is deur radioligandbindingstudies bepaal. Die binding van [^{35} S]-GTP γ S aan G $_o$ -proteïen in die afwesigheid van 'n agonis voor en na behandeling met benekstramien is ook ondersoek.

Resultate verkry deur G $_i$ -gemedieerde effekte in α_{2A} -L-selle te meet suggereer dat fenoksibensamien (100 μ M, 20 minute) en benekstramien (10 μ M, 20 minute) onomkeerbare non-spesifieke antagonisme by α_{2A} -AR'e openbaar, maar nie die affiniteit vir α_{2A} -AR'e in α_{2A} -H-selle verander nie. Dit wil ook voorkom asof die waargenome onomkeerbare non-spesifieke antagonisme deur benekstramien tyd- en konsentrasie-afhanklik is. Toe die meganisme van onomkeerbare antagonisme deur benekstramien verder ondersoek is, is gevind dat benekstramien die binding van [^{35} S]-GTP γ S aan α_{2A} -H membrane met beskermde α_{2A} -AR'e verminder het, maar nie die binding van [^{35} S]-GTP γ S aan G $_o$ in die afwesigheid van 'n agonis nie. Dit is ook gevind dat benekstramien onomkeerbare non-spesifieke antagonisme openbaar deur die G $_s$ -gemedieerde effekte van α_{2A} -AR'e in α_{2A} -H-selle en die G $_q$ -gemedieerde effekte van mACh-R'e of 5HT $_{2A}$ -R'e in SH-SY5Y- of 5HT $_{2A}$ -SH-SY5Y-selle respektiewelik te verlaag. 4-DAMP mosterd (100 nM, 20 minute) het nie onomkeerbare non-spesifieke antagonisme by mACh-R'e in SH-SY5Y-selle openbaar nie, maar onomkeerbare non-spesifieke antagonisme is waargeneem toe die inkubasietyd verleng is (100 nM, 60 minute).

Dit is dus gevind dat fenoksibensamien, benekstramien en 4-DAMP mosterd onder tipiese eksperimentele toestande onomkeerbare non-spesifieke antagonisme openbaar. Hierdie bevindinge bevestig die besorgdhede in die literatuur en ondersteun die moontlikheid dat meer onomkeerbare antagoniste ook onomkeerbare non-spesifieke antagonisme kan openbaar, en dit kan die interpretasie van data beïnvloed wat met hierdie geneesmiddels verkry is. Benekstramien mag ook nuttig wees as 'n eksperimentele geneesmiddel om seingeleiding in G-proteïengekoppelde reseptore te bestudeer.

Sleutelwoorde: 4-DAMP mosterd; 5HT $_{2A}$ -serotonienreseptor; α_{2A} -adrenoseptor; benekstramien; fenoksibensamien; G-proteïengekoppelde reseptor; muskariniese asetielcholinreseptor; non-sintopiese bindingsetel; onomkeerbare antagonis.

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Preface

Format of this thesis

This thesis has been compiled in the article format, whereby the methods, results and discussions of this study were incorporated into two full articles (*Chapter 3* and *Chapter 4*) intended for submission for publication in accredited journals. A review article is represented in *Appendix A*. The "Instructions to the Authors" prescribed by the appropriate journal were followed, except for the numbering of headings. Figures and their corresponding legends were appropriately placed within the text and the headings numbered.

This thesis has been written in Oxford English (U.K.), except for *Chapter 4* that was written in American English (U.S.) since this chapter consists of an article intended for submission to a U.S. journal.

The references in *Chapter 1* and *Chapter 2* are cited according to the Harvard method prescribed by the Potchefstroom University for Christian Higher Education. The references of the papers represented in *Chapter 3*, *Chapter 4* and *Appendix A*, however, are cited according to the format prescribed by the appropriate journal.

Participation of authors in articles

The authors of the articles represented in this thesis have each contributed substantially to the following aspects:

Title: *The classical irreversible competitive antagonists phenoxybenzamine, benextramine and 4-DAMP mustard, display non-competitive antagonism*

Journal: *British journal of pharmacology*

Brink, C.B.:	Project conception, initiation and overall layout; advice and suggestions on broad experimental design, the interpretation of data and diverse theoretical and practical aspects; proofreading
Bodenstein, J.:	Literature survey; detailed experimental planning and performance; reporting of data, data analyses, statistical analyses, representation and interpretation of data; drafting; proofreading
Venter, D.P.:	Initial identification of the research problem, theoretical advice and suggestions; proofreading

Title: *Benextramine is an irreversible non-specific inhibitor of several G protein-coupled receptors that signal through G_i , G_s and G_q*

Journal: *Molecular pharmacology*

Brink, C.B.:	Project conception, initiation and overall layout; advice and suggestions on broad experimental design, the interpretation of data and diverse theoretical and practical aspects; proofreading
Bodenstein, J.:	Literature survey; detailed experimental planning and performance; reporting of data, data analyses, statistical analyses, representation and interpretation of data; drafting; proofreading
Venter, D.P.:	Initial identification of the research problem, theoretical advice and suggestions; proofreading

Title: *Recent advances in drug action and therapeutics: Relevance of novel concepts in G protein-coupled receptor and signal transduction pharmacology*

Journal: *British journal of clinical pharmacology*

Brink, C.B.:	Project conception and initiation; writing; drafting; proofreading
Harvey, B.H.:	Specialist contributions and writing; proofreading
Bodenstein, J.:	Specialist contributions and writing; proofreading
Venter, D.P.:	Specialist contributions and writing; proofreading
Oliver, D.W.:	Specialist contributions and writing; proofreading

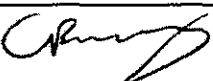
Approval for submission

The authors of the articles represented in this thesis have given their approval that the articles may be used for the purposes of this study.

Bodenstein, J.:



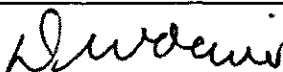
Brink, C.B.:



Harvey, B.H.:



Oliver, D.W.:



Venter, D.P.:



Introduction

Chapter 1

1.1 Problem statement

The present pharmacological study focused on the mechanism(s) of antagonism by a group of drugs classified as irreversible (non-equilibrium, in the kinetic sense) antagonists. The investigation involved a synergistic combination of classical pharmacological and biomolecular (subcellular signal-transductional) approaches. In general the elucidation of the biomolecular mechanisms of drug action, being a subspeciality of pharmacodynamics, is considered to be the most fundamental speciality in pharmacology (Gilman, 2001). It not only contributes to our understanding of drug action and to classify therapeutic agents more appropriately, but also to the development of new types of drugs with enhanced therapeutic efficacy and/or improved tolerability in humans. Not all drugs have direct therapeutic applications, and some are most useful as *in vitro* experimental tools to investigate the pharmacodynamic properties of drugs, or the biological systems (normal and pathological) in which drugs operate.

Experimental observations suggest that many irreversible antagonists inhibit pharmacological receptors (i.e. receptors with syntopic or orthosteric binding sites on the receptor macromolecule that bind agonists (Jenkinson, 2003)) in a time- and concentration-dependent manner (Furchgott, 1954 and Nickerson, 1956), presumably by binding to the receptor with a strong covalent bond (Ariëns *et al.*, 1960). Thereby the drug-receptor binding is irreversible with normal washing procedures, so that the receptors bound by the irreversible antagonist are rendered inoperative (frequently referred to as inactivated receptors). Consequently the number of available (operative)

receptors is reduced, while the bound receptors are unable to interact with an agonist to elicit a pharmacological effect¹.

Since irreversible antagonists inactivate pharmacological receptors, they have been extensively employed in the experimental pharmacology to investigate for example spare receptors (also referred to as receptor reserve, spare capacity or non-linear stimulus-effect relationship – see below) and to determine the relative efficacy of full agonists in systems displaying spare receptors (Barlow *et al.*, 1991; Brink *et al.*, 2000; Eglen & Harris, 1993; Furchgott, 1966; Morey *et al.*, 1998 and Van der Graaf & Stam, 1999).

A non-linear relationship between observed pharmacological effect and stimulus (as represented by agonist binding) is a common and well-known phenomenon in receptor pharmacology and has been explained classically by introducing the concept of spare receptors (Stephenson, 1956). This concept is further discussed in *Chapter 2*.

Consequently, a common application of irreversible antagonists has been to eliminate or reduce spare receptors in an attempt to investigate this phenomenon and the relative intrinsic efficacies of agonists where spare receptors presumably induce non-linearity between stimulus and effect. One approach is to utilise the classical Furchgott analysis that compares concentration-effect² curves before and after pre-treatment with an irreversible antagonist (Furchgott, 1966).

While based on the classical occupation theory, the Furchgott analysis only assumes that equal submaximal agonist-induced effects before and after pre-treatment of pharmacological receptors with the irreversible antagonist result from equal stimuli (i.e. conditions of equal receptor occupancy), and that the irreversible antagonist has only reduced the receptor concentration without modulating signal transduction through, or binding affinity for the agonist at the remaining fraction of operative receptors (Furchgott, 1966).

However, in addition to inactivating pharmacological receptors and modulating agonist effects by binding to syntopic binding sites, an irreversible antagonist may

¹ Although the term "response" is frequently used in the literature to denote a pharmacological effect, the term "effect" is used for the purposes of this thesis.

² Although the term "dose" is frequently used in the literature to denote a drug concentration, the term "concentration" is used for the purposes of this thesis.

theoretically also interact with other different binding sites on the same receptor macromolecule that do not compete with agonist binding (Jenkinson, 2003). These binding sites can be referred to as non-syntopic binding sites of the receptor macromolecule. This possibility has not been investigated extensively, despite data and suggestions in literature supporting the idea that irreversible antagonists may also display non-specific (allosteric, allotopic or non-competitive) mechanisms of antagonism by binding to non-syntopic binding sites or involve a molecular locus distinct from the receptor:

1. The primary concerns originated from observations by Van Ginneken (1977) who studied the irreversible antagonist dibenamine on the isolated guinea-pig ileum. He observed that, although pre-treatment with dibenamine resulted in a decrease in the maximal isometric contraction obtainable with the muscarinic acetylcholine receptor agonist methylfurfurethonium, the rate (measured in seconds) at which maximal contraction was reached for the same concentration was faster than in the absence of dibenamine. To explain his observations, he reasoned that dibenamine possibly changes the rate constants involved in the kinetics of drug-receptor interaction, caused by a conformational change in the receptor.
2. Van der Graaf *et al.* (1996) investigated irreversible non-specific antagonism of the irreversible antagonist benextramine at prostanoid TP-receptors. They observed that neither a relatively high concentration of the prostanoid TP-receptor agonist U46619, nor of the competitive antagonist SQ 30,741, could protect against the irreversibly antagonistic properties by benextramine against prostanoid TP-receptor agonist-mediated effect. To explain these observations, Van der Graaf *et al.* (1996) reasoned that benextramine does not bind to the same receptor site (syntopic binding site) as the agonist. However, they did not investigate the mechanism of the non-specific antagonism.

3. Brink *et al.* (2000) utilised the Furchgott analysis by employing different concentrations of benextramine to inactivate α_{2A} -adrenoceptors and obtain estimates of the relative efficacies of a series of α_{2A} -adrenoceptor agonists. However, they found that after pre-treatment with the higher concentration of benextramine used, the estimated apparent K_A values³ of UK 14,304 at α_{2A} -adrenoceptors, as calculated from submaximal concentration-effect curves, were between three- and five-fold higher than the calculated K_i value. They concluded that non-specific antagonism may be involved when the higher concentration of benextramine was used at the experimental conditions that they used.
4. Brink (1997) and Bodenstein (2000) also pointed out the probability and possibility of irreversible non-specific antagonism by several irreversible antagonists. The analysis of experimental data obtained from literature as well as from experiments conducted on isolated animal organs showed that the Hill slopes of concentration-effect curves changed significantly after treatment with several irreversible antagonists. Importantly, they also reasoned that the low level of receptor selectivity of some of the irreversible antagonists (e.g. phenoxybenzamine that inhibits adrenoceptors, muscarinic acetylcholine receptors and serotonergic receptors) enhances the likelihood that the drug also binds to other binding sites such as non-synaptic binding sites. They also noted that irreversible antagonists in general are known to be chemically highly reactive and are able to interact covalently with many entities that possibly include non-synaptic binding sites. These observations suggest that irreversible antagonists may modulate signal transduction by altering the receptor-effector coupling.

In conclusion, irreversible antagonists are still used in various applications to reduce or eliminate spare receptors despite of insufficient data to confirm that they do so only in a specific manner (i.e. excluding significant non-specific antagonism). If an

³ K_A and K_i values refer to estimates of the K_D value as obtained, respectively, from the Furchgott analysis of functional data or from competition binding data. The K_D value of a particular ligand at its receptor refers to the equilibrium dissociation constant of the ligand-receptor complex. In this thesis, K_D values are reported as "the K_D value of the ligand at its receptor", where the aforementioned definition is implied. The pK_D , pK_A and pK_i values refer to the negative logarithm of the K_D , K_A and K_i values, respectively. These definitions were applied throughout the thesis.

irreversible antagonist is found to display irreversible non-specific antagonism, this may prompt for the reinterpretation of data from previous experiments.

1.2 Study objectives

The primary objectives of this study were to determine:

- whether the classical irreversible competitive antagonists phenoxybenzamine⁴, benextramine⁵ and 4-DAMP mustard⁶ display non-specific antagonism,
- and if so, what the possible nature of these non-specific mechanisms are, which may include:
 - non-specific allosteric antagonism whereby agonist affinity is decreased, or
 - non-specific signal transductional antagonism whereby receptor signal transduction is modulated.

It was expected that the results of the present study will contribute to existing knowledge, regarding the mechanisms of antagonism by phenoxybenzamine, benextramine and 4-DAMP mustard (as a selection of prototypes of irreversible antagonists). The demonstration of irreversible non-specific mechanisms of antagonism by irreversible antagonists may reveal possible erroneous applications of these drugs and possibly open the doors for new applications of irreversible antagonists.

1.3 Study approach

A pharmacological approach was followed to investigate the proposed irreversible non-specific antagonism by phenoxybenzamine, benextramine and 4-DAMP mustard. All experiments were conducted at the Cell Culture Laboratory of the School of Pharmacy (Division of Pharmacology), Potchefstroom University for Christian Higher Education, Potchefstroom, North-West Province, South Africa.

The following four cell lines were utilised to investigate drug action:

⁴ Phenoxybenzamine has been reported to bind irreversibly to α -adrenoceptors, D₁-dopamine receptors, H₁-histamine receptors, oxytocin receptors and muscarinic acetylcholine receptors – see *Section 2.3.2.1.2*.

⁵ Benextramine has been reported to bind irreversibly to 5HT_{1A}-serotonin receptors, α -adrenoceptors, H₂-histamine receptors and neuropeptide Y-receptors – see *Section 2.3.2.2.2*.

⁶ 4-DAMP mustard has been reported to bind irreversibly to muscarinic acetylcholine receptors – see *Section 2.3.2.3.2*.

- the first two cell lines were derived from the Chinese hamster ovary CHO-K1 cell line, transfected to express relatively high numbers (designated α_{2A} -H cells) or lower numbers (designated α_{2A} -L cells) of the wild type porcine α_{2A} -adrenoceptor,
- a human neuroblastoma SH-SY5Y cell line that endogenously expresses M_1 , M_2 , and predominantly M_3 -muscarinic acetylcholine receptors, and
- SH-SY5Y cells transfected to express the human $5HT_{2A}$ -serotonin receptor (designated $5HT_{2A}$ -SH-SY5Y cells).

The experiments consisted of functional assays and radiolabelled ligand-binding assays. In the functional assays, drug action was investigated by measuring the whole-cell accumulation of the following second messengers:

- [^3H]-cAMP accumulation in α_{2A} -L cells to investigate the antagonism by benextramine, and
- [^3H]-IP_x⁷ accumulation in either SH-SY5Y or $5HT_{2A}$ -SH-SY5Y cells to investigate the antagonism by benextramine, and in SH-SY5Y cells to investigate the antagonism by 4-DAMP mustard.

To further investigate the antagonism by benextramine at subcellular level, functional assays also included the measurement of [^{35}S]-GTP γ S binding to membranes prepared from α_{2A} -H cells, or binding to the purified $G_o\alpha$ guanine nucleotide-binding regulatory protein.

Drug action was investigated on whole cells by conducting the following radioligand binding assays:

- Saturation binding assays were conducted in either α_{2A} -H cells to determine the α_{2A} -adrenoceptor concentration (B_{max} value) and the K_D value of [^3H]-yohimbine at α_{2A} -adrenoceptors, or in $5HT_{2A}$ -SH-SY5Y cells to determine the

⁷ IP_x (or IP_s) refers to the total phosphorylated inositolphosphates. In the Ca^{2+} -phosphoinositide signalling pathway, phospholipase C (PLC) hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP₂) to the second messengers *sn*-1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). The latter is rapidly dephosphorylated to IP₂ and IP, where the dephosphorylation of IP₂ and IP is prevented by the addition of lithium (Jenkinson, 1995). The assay that was used separates the accumulated phosphorylated forms of inositol (predominantly IP, IP₂ and IP₃ and their respective isoforms) from other products, where the phosphorylated forms of inositol are collectively referred to as IP_x. The measured quantity of IP_x is quantitatively related to the IP₃ synthesised upon activation of PLC.

5HT_{2A}-serotonin receptor concentration ($B_{\max}$ value) and the K_D value of [³H]-spiperone at 5HT_{2A}-serotonin receptors.

- Competition binding assays were conducted in α_{2A} -H cells to determine the K_i value of UK 14,304 at α_{2A} -adrenoceptors before and after the appropriate pre-treatment with an irreversible antagonist, and thereby to investigate the mechanism of antagonism by benextramine and phenoxybenzamine.
- Single-concentration radioligand binding assays were conducted to determine relative receptor concentrations before and after the appropriate pre-treatment with an irreversible antagonist. The radioligands used included [³H]-yohimbine at α_{2A} -adrenoceptors in α_{2A} -H cells, [³H]-4-DAMP at M₃-muscarinic acetylcholine receptors in SH-SY5Y cells and [³H]-ketanserin at 5HT_{2A}-serotonin receptors in 5HT_{2A}-SH-SY5Y cells.

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The Theory and Application of Irreversible Antagonists

Chapter 2

Irreversible antagonism is central to the theme of this thesis and is therefore discussed in more detail. This literature survey discusses additional aspects of irreversible antagonists that are not discussed in detail in the papers presented in *Chapter 3*, *Chapter 4* and *Appendix A*.

2.1 Introduction

Jenkinson (2003) defines an irreversible antagonist as *"a drug that forms a long-lasting or even irreversible combination with either the agonist binding site or a region related to it in such a way that agonist and antagonist molecules cannot be bound at the same time"*. However, although this definition states that the irreversible antagonist binds to the same or a related binding site as the agonist¹ and thereby inhibits the subsequent binding of an agonist, it does not include the possibility of non-specific mechanisms such as signal-transductional antagonism when the irreversible antagonist may bind irreversibly to other binding sites² on the receptor macromolecule (see below). Also, competitive antagonist ligands do not necessarily interact with the same binding site as agonist ligands. This has been shown for non-peptide antagonists of tachykinin receptors (Gether *et al.*, 1993), tramadol and lidocaine of Ca²⁺ in nerve conduction (Mert *et al.*, 2001), peptidic and non-peptidic antagonists of neuropeptide Y receptors (Kannoa *et al.*, 2001). Practically, a drug is considered to be an irreversible antagonist when the antagonism that is observed with the drug cannot be reversed after washing the pharmacological system with a drug-free solution (Kenakin, 1997).

¹ The agonist binding site in the receptor macromolecule is referred to as a syntopic binding site for the purposes of this thesis.

² A binding site that is different from the agonist binding site on the same receptor macromolecule and is referred to as a non-syntopic binding site for the purposes of this thesis. However, additional mechanisms of actions of irreversible antagonists may not only involve non-syntopic sites, but may also involve a molecular locus distinct from the receptor macromolecule, such as a G protein.

However, present knowledge is too limited to comprehend the entire mechanism of action of irreversible antagonists.

Irreversible antagonism, and especially the application of irreversible antagonists to investigate non-linearity between stimulus and effect, has been an important field of research since the early 1950s, when a number of irreversible antagonists with activity at various receptor types were synthesised and characterised. The unique properties of irreversible antagonists to inactivate pharmacological receptors (see below) resulted in these drugs being extensively employed in the experimental pharmacology to investigate many drug-receptor interactions. However, besides its experimental applications, phenoxybenzamine is an example of an irreversible antagonist that has been used clinically for the treatment of essential hypertension since the early 1950s until later in the 20th century and is still considered to be the primary drug in the treatment of phaeochromocytoma. Some drugs may also bind irreversibly to enzymes and can be considered as irreversible antagonists. An example is ecothiophate, an anticholinesterase drug that is related to the organophosphates and employed to treat advanced glaucoma. However, this study focuses on non-specific mechanisms of antagonism by irreversible antagonists that primarily interact with pharmacological receptors, such as α_{2A} -adrenoceptors.

In this chapter, *Section 2.2* deals with the theoretical background of a few concepts considered to be indispensable to understand the concept and scope of irreversible antagonism. The pharmacology and experimental application of a selection of classical irreversible antagonists that have also been employed in the current study, are discussed in *Section 2.3* and a few concluding remarks are reported in *Section 2.4*.

2.2 Theoretical background

2.2.1 Receptors and ligands

Jenkinson (2003) defines the concept "receptor" as it is used in current receptor pharmacology to *"denote a class of cellular macromolecules that are concerned specifically and directly with chemical signalling between and within cells"*. Therefore, the two primary functions of receptors are to recognise the particular molecules

(ligands) that activate distinct regions thereof (syntopic binding sites in the case of agonists), and when recognition occurs, to modulate cell function (e.g. by promoting or inhibiting the formation of second messengers by modulating ion channels or by altering gene transcription).

Progresses in the techniques of molecular biology have revealed the amino acid sequence of an increasing number of signalling molecules (e.g. receptor macromolecules containing essential binding sites for ligands). Consequently, particular regions in these receptor macromolecules have been identified that play important roles in the ligand binding and receptor signalling (Baldwin *et al.*, 1997; Ikezu *et al.*, 1992; Okamoto & Nishimoto, 1992; Strader *et al.*, 1987; Wade *et al.*, 1999). For example, the amino acid sequences for the porcine α_{2A} -adrenoceptor (Guyer *et al.*, 1990), human M_3 -muscarinic acetylcholine receptor (Peralta *et al.*, 1987) and human 5HT₂-serotonin receptor (Saltzman *et al.*, 1991) have been determined. The functional domains on a receptor macromolecule usually include most prominently the ligand-binding domain(s) (i.e. syntopic binding site(s) for an agonist and possible non-syntopic binding sites – see below) and the effector domain that transfers the signal to a second entity (e.g. G proteins).

Jenkinson (2003) defines a syntopic binding site (also referred to as an orthosteric- or specific binding site (Haylett, 2003)) as a binding site on the receptor macromolecule that has affinity for a ligand that can be an agonist or antagonist. When an agonist binds to the syntopic binding site it elicits a measurable pharmacological effect, whereas when an antagonist (specifically a neutral competitive antagonist) binds to the syntopic binding site, no effect is observed. When an inverse agonist binds to a receptor it reduces any basal effect and may therefore elicit an effect opposite to that of the agonist in an appropriate pharmacological system (see a more comprehensive discussion in *Appendix A*). In addition, the binding of agonist and competitive antagonist to syntopic binding sites is mutually exclusive, since per definition they compete with each other for binding and cannot bind to one syntopic binding site simultaneously. Alternatively, binding of the competitive antagonist to the receptor, prevents binding of the agonist to the syntopic binding site and *vice versa*. For the purposes of the present study, syntopic binding sites refer to those binding sites on the

receptor macromolecule that are able to bind an agonist and elicit a specific biological (or pharmacological) effect.

Syntopic binding sites should be distinguished from allotopic binding sites. Jenkinson (2003) defines an allotopic binding site (also referred to as an indirect, allosteric, non-competitive or non-specific binding site (Haylett, 2003)) as a distinct binding site on the receptor macromolecule that does not have affinity for an agonist, but for a non-specific ligand. According to Jenkinson (2003), the binding of the agonist and the non-specific ligand is not mutually exclusive, since both do not compete with each other for binding, but they bind to different binding sites – the agonist to a specific binding site, and the ligand to a non-specific binding site. However, Ariëns *et al.* (1964a) mentioned the theoretical possibility that an agonist may, besides binding to the specific binding site, also bind to additional (non-specific) binding sites and thereby inhibit its own effects (non-competitive auto-interaction).

For the purpose of the present study, non-syntopic binding sites refer to those binding sites on the receptor macromolecule that do not elicit a pharmacological effect upon binding to an agonist. These sites are able to bind to ligands (e.g. non-specific antagonists) in a saturable manner that may either:

- **induce a conformational change in the receptor macromolecule to modulate the affinity of the syntopic binding site for its ligands (allosteric binding sites)³, or**
- **modify the receptor-effector coupling (signal transduction system) of the specific receptor and modulate signal transduction within the cell⁴.**

It has been mentioned above that ligands bind to binding sites on receptor macromolecules. Jenkinson (2003) defines a ligand as a relatively small molecule (when compared to the size of the receptor macromolecule) that may bind to one or more binding sites on the receptor macromolecule.

³ Ariëns *et al.* (1956) used the term "metaffinoid antagonism" to describe this type of non-specific antagonism.

⁴ Ariëns *et al.* (1956) used the term "metactoid antagonism" to describe this type of non-specific antagonism.

Considering the different types of binding sites on the receptor macromolecule and the different functions of ligands described above, the following definitions are important for the present study:

- An agonist is defined as a ligand that binds to a syntopic binding site on the pharmacological receptor to elicit a pharmacological effect.
- A competitive antagonist⁵ is defined as a ligand that binds to a receptor without eliciting a pharmacological effect, and may compete with an agonist for binding to the same binding site on the receptor. The antagonist can bind reversibly or irreversibly to the specific binding site.
- A non-specific antagonist is defined as a ligand that binds to a non-syntopic binding site. It does not elicit a pharmacological effect and does not compete for binding with an agonist, but either modulates the signal transduction of the pharmacological receptor or affects ligand binding to the receptor. The non-specific antagonist can bind reversibly or irreversibly to the non-syntopic binding site.

It is evident that the different ligands defined above may bind to different binding sites on the receptor macromolecule. A large and important family of cell-surface receptor macromolecules are known as guanosine triphosphate nucleotide (GTP) binding protein-coupled receptors (commonly abbreviated as GPCRs). The GPCRs are also known as seven transmembrane (7TM) receptors, referring to their macromolecular structure spanning the cell membrane seven times. GPCRs activate heterotrimeric G proteins to mediate biological responses (Gudermann *et al.*, 1997) by means of signalling processes, second messenger regulation, ion channel modulation, cell growth and differentiation, and cross-talk with tyrosine kinases and small molecular-weight G proteins.

GPCR allosterism and complexing have been extensively reviewed by Christopoulos & Kenakin (2002). GPCRs are classified as natural allosteric proteins since agonist-mediated signalling requires a conformational change in the receptor protein transmitted between two topographically distinct binding sites, namely one for the agonist (orthosteric site) and one for the G protein. However, evidence suggests that

⁵ Inverse agonism will not be considered in this study and competitive antagonism is considered as neutral antagonism.

the agonist-bound GPCR can also form ternary complexes with other ligands or "accessory" proteins (allosteric site) and thereby displays altered binding and/or signalling properties in relation to the binary agonist-receptor complex. Allosteric binding sites on GPCRs represent novel drug targets and are of a non-competitive nature. Moreover, current knowledge regarding the location of possible allosteric binding sites is limited and the ligand binding properties thereof not always well understood. However, the most important application of knowledge obtained with the study of allosteric phenomena is the discovery of new drugs.

The best-studied example of GPCRs is the rhodopsin receptor. A computer-generated reconstruction of the bovine rhodopsin receptor is depicted in *Figure 2-1* (Schwartz & Holst, 2003). The model is based on three-dimensional crystallography and shows the receptor in its presumed inactive (non-signalling) conformational state (see *Appendix A* for discussion of the multiple states of receptor activation). The rhodopsin receptor in the rod cells of the retina in the human eye is responsible for vision when the ligand 11-*cis*-retinal is chemically isomerised by photo-energy (light focused on the retina) to all-*trans*-retinal (Vander *et al.*, 1994).

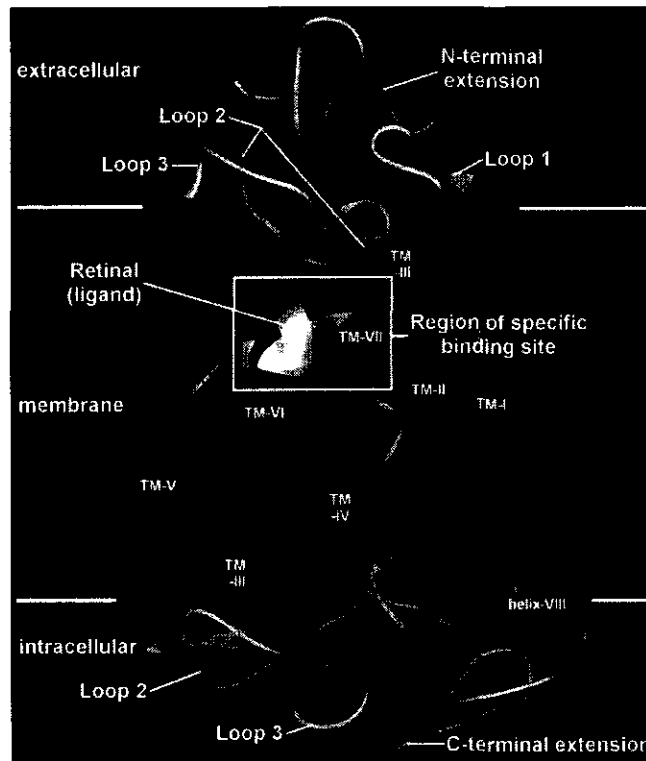


Figure 2–1: A computer-generated x-ray structural model of the bovine G protein-coupled receptor (GPCR), rhodopsin, in its inactive state. Note the seven transmembrane (7TM) helices, intracellular and extracellular connecting loops, and the binding of the chromophore ligand 11-*cis*-retinal. 7TM GPCRs include a large family of receptors, with classical examples such as the α -adrenoceptors, muscarinic acetylcholine receptors, and serotonergic receptors. This figure illustrates the concepts receptor (as the macromolecule), binding site (that can either be syntopic (specific) or non-syntopic (non-specific)), and ligand (an agonist or antagonist). An agonist binds per definition to a syntopic binding site on the receptor macromolecule. Figure adapted from Schwartz & Holst (2003).

2.2.2 Agonist-mediated stimulus and efficacy

The pharmacodynamic characteristics of an agonist can best be described in terms of affinity and efficacy. Affinity refers to the property of attraction of the agonist to bind to a pharmacological receptor (sum total of binding forces), whereas efficacy describes the ability of an agonist to elicit a pharmacological effect at a pharmacological receptor. An understanding of the terms and concepts "efficacy" and "stimulus" are important for understanding the concept "non-linear relationship between stimulus and effect", which in turn is important to understand the application of irreversible antagonists. For this reason "efficacy" and "stimulus" are important concepts also for the current study and their meanings are discussed briefly.

Ross & Kenakin (2001) defines efficacy as *"the information encoded in the drug's chemical structure that causes the receptor to change accordingly when the drug is bound"*. According to Ariëns (Mackay, 1977) the pharmacological stimulus is considered to be the primary effect in the sequence of events before a pharmacological effect is actually measured. Stephenson (1956) defined it mathematically as the product of the efficacy of an agonist and the fraction of the specific receptors bound. The stimulus obtained after an agonist has occupied a specific receptor is conveyed to physiological effectors by biochemical reactions to elicit a pharmacological effect. Ross & Kenakin (2001) describe efficacy further as *"a function of occupancy and the stimulus-effect function (comprising all of the biochemical reactions that take place to translate agonist binding into effect) and amplifies stimulus"*. When Furchgott (1965) recognised that the pharmacological effect of an agonist is influenced by the total receptor concentration and the efficacy, he introduced the term intrinsic efficacy that is defined as the stimulus per agonist-receptor complex. In contrast to efficacy, intrinsic efficacy is therefore considered to be a drug property at a particular receptor. However, recent studies suggest that intrinsic efficacy may also be dependent on the receptor signalling pathway for a given agonist (Berg *et al.*, 1998; Brink *et al.*, 2000; Kenakin, 1995). The intrinsic efficacy of one agonist can be expressed in terms of the intrinsic efficacy of a reference agonist (usually the endogenous or a strong agonist), referred to as relative intrinsic efficacy. It is possible to classify an agonist according to its relative intrinsic efficacy at its receptor.

Relative intrinsic activity was originally introduced by Ariëns (1954), and is now usually defined as the maximal pharmacological effect of an agonist relative to that of a full agonist acting through the same receptors in the same pharmacological system (Jenkinson, 2003). Relative intrinsic activity, which differs from intrinsic efficacy, is also dependent on the properties of the pharmacological system in which the effect is determined.

2.2.3 Non-linear stimulus-effect relationship

Non-linear relationship between stimulus and effect has been a topic of investigation and debate for many decades. Clark (1937) introduced the classical receptor occupation theory in an attempt to mathematically describe agonist-receptor

interactions. This model was later modified by Ariëns (1954), Stephenson (1956), Van Rossum & Ariëns (1962) and Furchgott (1966) after observations by Raventós (1937) and Nickerson (1956). Ariëns (1954) assumed that the stimulus-effect relationship is linear, but this assumption was later eliminated (Van Rossum & Ariëns, 1962). Stephenson (1956) suggested that, for some agonists in some pharmacological systems the relationship between the receptor binding (or stimulus) and the effect is not linear, since maximal effects were obtained before all receptors were bound by the agonist (i.e. maximal effects were obtained at submaximal stimuli). In an attempt to account for non-linearity in the signal transduction system, Stephenson (1956) introduced the concept of spare receptors. Therefore, an agonist with high efficacy in a given pharmacological system may need to occupy only a fraction of all the receptors to elicit a maximal pharmacological effect.

Briefly, spare receptors (also termed receptor reserve, spare capacity or non-linear stimulus-effect relationship) are those receptors that are unbound (in excess) at the lowest concentration of an agonist that produces maximal effect. The presence of spare receptors is qualitatively and quantitatively dependent on both the biological (pharmacological) system and the agonist under investigation. For example, in a given pharmacological system with a relatively high receptor concentration, agonist A with high efficacy may be a full agonist with significant spare receptors, while agonist B with low efficacy may act as a partial agonist with no spare receptors (qualitative difference). In the same system agonist C with lower efficacy than agonist A, but higher efficacy than agonist B, may also be a full agonist, but with less spare receptors than agonist A (quantitative difference between the spare receptors for agonists A and C). In a different pharmacological system with an even higher receptor concentration, agonists A, B and C may all act as full agonists with spare receptors, and in a system with a relatively low receptor concentration none of the agonists may have spare receptors.

Another explanation for a non-linear relationship between stimulus and effect has been provided by Ariëns (1964b), where he proposed that certain agonists may elicit effects only after a certain threshold stimulus has been reached. Ross & Kenakin (2001) explain a non-linear relationship between stimulus and effect in terms of the efficiency of the stimulus-effect coupling.

The Furchgott analysis (see *Section 2.3.1*) has been employed to estimate the relative intrinsic efficacies of a series of agonists in a system with spare receptors.

2.2.4 Irreversible antagonism

Irreversible antagonism was defined in *Section 2.1*. The magnitude of the antagonism (amount of receptors bound irreversibly) is dependent on both the incubation time with the irreversible antagonist and the concentration of the irreversible antagonist (Furchgott, 1954; Nickerson, 1956). Consequently, the irreversibly bound receptors are rendered inoperative (also referred to as inactivated receptors) and they are unable to bind to an agonist and elicit a pharmacological effect (Jenkinson, 2003). In addition, it has been proposed that some irreversible antagonists may bind irreversibly to non-specific binding sites, whereby it is possible to interfere with receptor signalling or to modulate the affinity of the syntopic binding sites for their ligands, such as agonists. The antagonism by irreversible antagonists has been described as "non-equilibrium blockade" (Nickerson, 1956), "unsurmountable blockade" (Gaddum *et al.*, 1955), and (currently) as "insurmountable" due to the following reasons:

- After an animal or tissue has been pre-treated with the irreversible antagonist at a specific concentration and incubation time, normal agonist-induced effects cannot be restored, even after washing the effector several times with drug-free solution and allowing sufficient equilibration time (Ariëns *et al.*, 1964b).
- After pre-treatment with the irreversible antagonist, relatively high concentrations of an appropriate agonist cannot surmount the antagonism (Ariëns *et al.*, 1964b).
- In the kinetic sense, the binding of the irreversible antagonist does not reach thermodynamic equilibrium (Melchiorre, 1981). Association (binding) takes place between molecules of the irreversible antagonist and binding sites on the receptor macromolecule, but little or no dissociation occurs (see below) (Chabner *et al.*, 2001). This proposal implies that the dissociation rate of the irreversible antagonist-receptor complex is insignificant when compared to the corresponding association rate and, according to Salmon & Sartorelli (2001), it

provides a possible explanation for the time-dependency of the magnitude of irreversible antagonism⁶.

- Presumably the dissociation of the irreversible antagonist-receptor complex is inhibited since strong covalent bonds may be formed between molecules of the irreversible antagonist and the chemical groups (e.g. –SH, –OH, =NH and –COOH) of amino acid residues of the receptors (Ariëns *et al.*, 1964b). However, according to Jenkinson (2003), not all irreversible antagonists interact with receptors by forming covalent bonds. Some may fit the binding site so well that the combined strength of weaker intermolecular interactions (e.g. ionic, hydrophobic, Van der Waals and hydrogen bonds) that come into play approaches the strength of a covalent bond. Consequently, the binding between irreversible antagonist and receptors changes the original stereochemical properties of the receptor macromolecule irreversibly. Since pharmacological receptors are thereby "inactivated", it may not be possible to elicit an agonist-induced pharmacological effect.

Although the antagonism of irreversible antagonists is insurmountable, the pharmacological effect to agonists can be restored in the living organism after new specific receptors have been synthesised (Jenkinson, 2003).

Recent models and methods for studying insurmountable antagonism have been reviewed by Vauquelin *et al.* (2002). Insurmountable antagonism is not only drug-related but also depends on the tissue, species and experimental design. Thereby many different theoretical models have been proposed to explain the operative mechanism. Also, the molecular mechanism for insurmountable antagonism is still a matter of debate.

First-generation models attempt to explain the non-competitive behaviour of insurmountable antagonists, as opposed to the competitive behaviour of surmountable antagonists. The theoretical model of Ariëns *et al.* (1956) classifies non-competitive antagonists as drugs that could interact with distinct binding sites to modulate the agonist-induced effect without affecting the agonist binding, and thereby inhibit effects

⁶ It should be noted that irreversibility in a pharmacological response depends not only on the ligand properties but also on the time-scale of the biological measurement. For example, what is effectively irreversible for an ion channel measurement that takes place over 100 milliseconds could easily be fully reversible for an enzyme assay that takes place over 1 hour.

from different receptor types. In addition, non-competitive antagonism could also take place when the antagonist binds to an allosteric site on the receptor, and thereby induces a conformational change in the receptor macromolecule that comprises its interaction with the agonist and/or its ability to elicit an effect.

Since it has been observed that many insurmountable antagonists exhibit mixed properties with only a partial depression of the maximal effect along with a rightward shift of the concentration-effect curve, the theoretical model by Ariëns *et al.* (1956) was extended to permit insurmountable antagonists and agonists to compete for the agonist binding site along with non-competitive antagonism via another (allosteric) binding site on the receptor (Ariëns *et al.*, 1964a). According to this model there are two inactive states of the receptor, the first is a thermodynamically stable conformation (R) that can be recognised and stimulated by an agonist to yield an activated agonist-receptor complex (AR*), and the second is a conformation (R') that is totally refractive to stimulation or unable to elicit an effect. This concept complies with current theories proposing multiple states of activity (conformational states) for a receptor. With insurmountable antagonism, equilibrium is driven towards R', and equilibrium differs from one antagonist to another. However, the major differences between the models deal with the explanations about how R' is produced, and the meaning thereof in molecular terms.

Allosteric interactions are discussed in the Kaumann-model (Kaumann & Frenken, 1985). In this model, antagonists modulate the interconversion between R and R' by binding to an allosteric binding site (E) on the receptor macromolecule that is different from the agonist binding site on the same receptor. With surmountable antagonists, the R:R' equilibrium is adjusted in favour of R, or in favour of R' with insurmountable antagonists. Both states coexist with partially insurmountable antagonists. According to this model, the maximal agonist effect is dictated by the ratio of R:R_{total}, and therefore the effect is only reduced to a limited extent by the mixed properties (see above) of such antagonists. In addition, all antagonists compete with E and this could give a possible explanation for the counteracting effects of surmountable and insurmountable antagonists. Thus, agonists, but also surmountable and insurmountable antagonists, interact with R. An interesting characteristic of this model is that the agonist binding site and E have common structural properties, and there is increasing

evidence that besides 5HT₂-serotonin receptors, for which this model was developed, other 7TM GPCRs can also form homodimeric complexes with two related binding sites (Kaumann & Frenken, 1985).

Another variation on the Kaumann model is that agonists and antagonists bind to the same or an overlapping site on the receptor. Again this attempts to explain the counteracting effects of surmountable and insurmountable antagonists. This allows R to form fast reversible and surmountable complexes with all antagonists (L). LR' complexes can be formed either by antagonist-induced $LR \leftrightarrow LR'$ isomerisation or, in the case of spontaneous $R \leftrightarrow R'$ isomerisation, by preferential binding of the antagonist to R'. Therefore, insurmountable antagonism can be described by the slow dissociation of L from LR', or in the case of fast-dissociating complexes, by the slow reconversion of R' into R. In addition, the antagonist does not need to stay bound to elicit a long-lasting effect (Kaumann & Frenken, 1985).

2.3 Irreversible antagonists

2.3.1 Applications

The unique properties of irreversible antagonists to inactivate receptors have resulted in these drugs to be extensively employed in the experimental pharmacology. Kenakin (1997) states that irreversible antagonists can be used as "chemical scalpels" to reduce the number of receptors, and at relatively higher concentrations, to eliminate spare receptors.

Jenkinson (1993) indicated that irreversible antagonists have been synthesised as radiolabeled ligands to investigate the type and number of receptors that an irreversible antagonist may interact with. For example, the neuromuscular endplate regions of patients suffering from the neuromuscular disease myasthenia gravis have been investigated with the radiolabelled irreversible antagonist [¹²⁵I]- or [¹³¹I]-α-bungarotoxin (one of many α-toxins identified in snake venom). This toxin binds irreversibly but non-covalently to nicotinic acetylcholine receptors. It was revealed that myasthenia gravis is caused by a reduction in the number of nicotinic acetylcholine receptors at the neuromuscular endplate regions in patients (Fambrough *et al.*, 1973).

Irreversible antagonists have been employed as a tool to selectively eliminate certain subtypes of receptors in a pharmacological system in order to examine the remaining receptors. This approach involves the use of a selective reversible competitive antagonist to protect the receptors of interest, followed by the addition of an irreversible antagonist to eliminate the remaining (unoccupied) receptors. After the removal of the irreversible antagonist and reversible antagonist by several washing steps and allowing ample dissociation time, the protected receptors can be selectively investigated by, for example, measuring agonist-induced pharmacological effects (Eglen *et al.*, 1994; Kenakin, 1997).

Antibodies that act as irreversible antagonists have also been synthesised and implemented to detect and map receptor types in pharmacological systems. Li *et al.* (1991) investigated the M₂-muscarinic acetylcholine receptor densities in the rat heart and in various regions of the rat brain. They identified a DNA fragment that encodes for a specific loop in the rat M₂-receptor and fused the fragment to the gene responsible for staphylococcal protein A. The fusion protein was administered to rabbits that raised a polyclonal antibody to the rat M₂-receptor. After pre-treating the various tissues with or without the antibody, the muscarinic acetylcholine receptor densities were determined from radioligand binding assays. By analysing differences in the binding of the radioligand before and after pre-treatment with the antibody, they were able to show that the muscarinic acetylcholine receptors in the rat heart are predominantly of the M₂ subtype, in contrast to the rat brain (cortex, hippocampus, striatum, olfactory tubercle, thalamus/hypothalamus, pons/medulla and the cerebellum).

Irreversible antagonists have been employed to reduce or eliminate spare receptors. The evidence that some full agonists may elicit maximal pharmacological effects without binding to all the available receptors may prove to complicate attempts to accurately estimate agonist-related parameters (e.g. affinity and efficacy), since the relationship between stimulus and effect in a given biological system is usually not known and difficult to determine (Mackay, 1977).

Stephenson (1965; 1966) proposed that the equilibrium dissociation constants of an agonist-receptor complex and the relative efficacy of an agonist can be estimated from concentration-effect curves by employing an irreversible antagonist. Stephenson (1956) based his proposal on the assumption that equal effects before and after partial

receptor inactivation by the irreversible antagonist result from equal agonist-induced stimuli (and therefore equal receptor occupancies). To obtain estimates for these agonist-related parameters, the null method approach for irreversible antagonism (also referred to as the Furchgott analysis) was mathematically derived by Furchgott (1966) and reviewed and discussed by Mackay (1966a; 1966b), Van Rossum (1966), Furchgott & Bursztyn (1967) and Waud (1968a; 1968b). The primary application of the Furchgott analysis is to determine estimates of the equilibrium dissociation constants for agonist-receptor complexes and thereby to classify agonists and receptors.

Briefly, the Furchgott analysis is based on the assumptions of the classical occupation theory of drug action originally proposed by Clark (1933). In addition, implicit to the Furchgott analysis is that the irreversible antagonist employed has not modified the stimulus-effect relationship by binding to non-syntopic binding sites. This analysis compares the corresponding agonist concentrations that result in equal submaximal effects being obtained before and after treatment with an irreversible antagonist. The primary function of the irreversible antagonist is to inactivate receptors or reduce the number of spare receptors, so that a higher concentration of agonist is needed to elicit the same effect obtained before pre-treatment with the irreversible antagonist. According to the theories of Stephenson (1956), these equal effects are assumed to be the result from equal induced stimuli, and are therefore equated.

The Furchgott analysis has been utilised extensively in the experimental pharmacology since its inception in the mid-1960s. Some examples, where different irreversible antagonists have been employed, include:

- phenoxybenzamine:
 - to determine estimated affinities for a series of muscarinic agonist-muscarinic acetylcholine receptor complexes in the isolated guinea-pig ileum (Eglen & Whiting, 1987), and
 - to determine estimates of the efficacy and affinity of noradrenaline- α -adrenoceptor complexes in the isolated rat small mesenteric artery (Van der Graaf & Stam, 1999).
- benextramine:
 - as part of a study to investigate agonist-directed trafficking. Brink *et al.* (2000) determined that the lower concentrations of benextramine at the

experimental conditions used does not significantly modulate the α_{2A} -adrenoceptor signal transduction, and

- to determine estimates of the relative intrinsic efficacies and affinities for a series of serotonergic agonist-5HT_{1A}-serotonin receptor complexes in human erythroleukemia cells (Stanton & Beer, 1997).
- 4-DAMP mustard:
 - to determine estimated affinities for a series of muscarinic agonist-muscarinic acetylcholine receptor complexes in the isolated guinea-pig ileum (Barlow *et al.*, 1991).

Other examples, illustrating the extensive use of irreversible antagonists *in vitro* in experimental pharmacology, include the classical α -adrenoceptor selective antagonist dibenamine (Siegl & McNeill, 1982), the α_{1B} -adrenoceptor selective antagonist CEC (chloroethylclonidine) (Giardinà *et al.*, 1995), the α_{2D} -adrenoceptor selective antagonist *N*-ethoxycarbonyl-1,2-dihydroquinoline (Tian *et al.*, 1996), the β -adrenoceptor selective antagonist Ro 3-7894 (Siegl & McNeill, 1982), the A₁-adenosine receptor selective antagonist FSCPX (8-cyclopentyl-3-[3-[[4-(fluorosulphonyl)benzoyl]oxy]propyl]-1-propylxanthine) (Morey *et al.*, 1998), the muscarinic acetylcholine receptor and 5HT_{1B}-serotonin receptor antagonist EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) (Adham *et al.*, 1993; Agneter *et al.*, 1993; Koek *et al.*, 2000) and the muscarinic acetylcholine receptor selective antagonist propylbenzilylcholine mustard (Eglen *et al.*, 1994).

2.3.2 Classical examples

The alkylating agents phenoxybenzamine and 4-DAMP mustard are chemically related to the nitrogen mustard compounds⁷ and share the same mechanism of action. Other examples of alkylating agents include some antineoplastic drugs⁸ (Salmon & Sartorelli, 2001). Phenoxybenzamine and 4-DAMP mustard cyclise in aqueous solutions after the loss of chloride ions and are converted to highly reactive

⁷ The prototype DNA alkylating agent is sulphur mustard, first synthesised in 1854. It was employed as chemical warfare agent during World War I, causing blisters on the skin, eyes and in the respiratory tract. This vesicant action was followed by serious systemic toxicity.

⁸ The bis(chloroethyl)amine compounds such as mechlorethamine (the prototype), cyclophosphamide (Endoxan®), chlorambucil (Leukeran®) and melphalan (Alkeran®) have been clinically employed in the treatment of certain types of cancer and autoimmune diseases.

ethyleneimmonium ions (also referred to as aziridinium ions) and carbonium ions (see *Figure 2–2*) (Salmon & Sartorelli, 2001).

Carbonium ions are able to form strong covalent bonds with electron donors, that include the $-SH$, $-OH$, $=NH$ and $-COOH$ chemical groups (Ariëns *et al.*, 1964b) of amino acid residues in receptors and proteins. These bonds can be formed between a molecule of the alkylating agent and the receptor or a related region in the receptor macromolecule or other molecular targets. Consequently, an agonist cannot bind to these binding sites to elicit a pharmacological effect (Jenkinson, 2003).

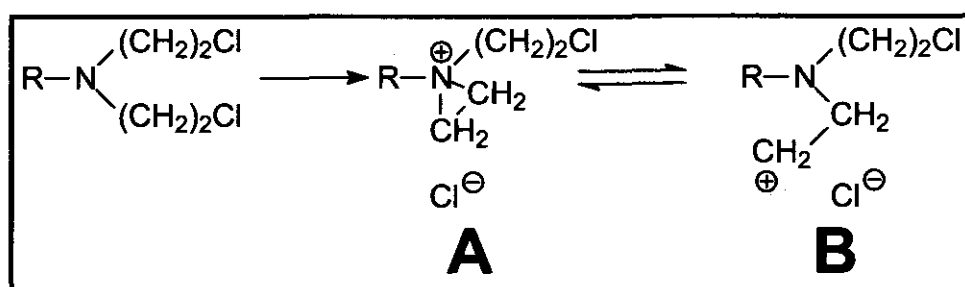


Figure 2–2: The alkylating agents (that include phenoxybenzamine and 4-DAMP mustard) cyclise in aqueous solutions after the loss of a chloride ion to yield highly reactive **(A)** aziridinium and **(B)** carbonium ions. Carbonium ions are capable of forming strong covalent bonds with nucleophilic moieties, such as the sulphydryl groups of certain amino acids present in proteins and receptors (Salmon & Sartorelli, 2001).

The tetramine disulphide benextramine, however, works differently and it has been proposed that benextramine inactivates receptors *via* a disulphide-thiol interchange reaction between the molecule and distinct binding sites on the receptor (Brasili *et al.*, 1986; Frang *et al.*, 2001; Giardinà *et al.*, 1996; Melchiorre, 1981).

2.3.2.1 Phenoxybenzamine

2.3.2.1.1 Chemical structure and properties

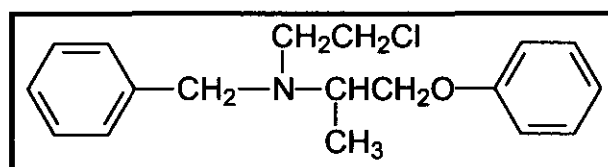


Figure 2–3: The chemical structure of phenoxybenzamine.

Phenoxybenzamine is chemically related to the prototype β -haloalkylamine dibenamine, one of a number of irreversible antagonists used to demonstrate the existence of spare receptors in a pharmacological system (Furchgott, 1954; 1955).

2.3.2.1.2 Receptor interactions

It has been reported that phenoxybenzamine is a multipotent irreversible antagonist since it interacts irreversibly with α_1 - and α_2 -adrenoceptors (Minneman, 1983), D_1 -dopamine receptors (Hall *et al.*, 1993), H_1 -histamine receptors (Jenkinson, 2003), muscarinic acetylcholine receptors (Eglen *et al.*, 1994), and oxytocin receptors (Rhee *et al.*, 1998).

2.3.2.1.3 Mechanism of action at α_{2A} -adrenoceptors

Frang *et al.* (2001) investigated the effect of phenoxybenzamine on the amino acid sequence and helical arrangement of the seven transmembrane domains of the α_{2A} -adrenoceptor. They determined the irreversible binding of phenoxybenzamine to the human α_{2A} -adrenoceptor wild-type and mutants in transfected Chinese hamster ovary cells. An α_{2A} -adrenoceptor mutant lacking a cysteine residue in the third transmembrane helix was constructed and tested (the cysteine was substituted with a valine residue). By conducting radiolabelled ligand binding assays, mass spectroscopic analysis and computational molecular modelling studies, they found that the alkylating properties of phenoxybenzamine are reduced in the absence of an accessible cysteine residue in the binding cavity of the third transmembrane helix (i.e. the α_{2A} -adrenoceptor mutant). The phenoxybenzamine-resistant mutant receptor that did not contain this cysteine residue was capable of binding phenoxybenzamine reversibly, but with lower apparent affinity than the sensitive receptors containing this residue. They concluded that the sulphydryl side chain of a cysteine residue in the third transmembrane domain of the α_{2A} -adrenoceptor is responsible for covalent binding of the aziridinium ion of phenoxybenzamine.

2.3.2.1.4 Experimental applications

Phenoxybenzamine is one of the irreversible multipotent antagonists that has been the most extensively employed in the experimental pharmacology, as confirmed by the

numerous related studies that have been published. For instance, phenoxybenzamine has been employed to inactivate α -adrenoceptors to determine the presence of spare receptors for agonists in various pharmacological systems. Fox & Friedman (1987) demonstrated the presence of spare α_1 -adrenoceptors for noradrenaline in the isolated vas deferens and caudal artery of the rat, and Guimaraes & Paiva (1987) for noradrenaline at α -adrenoceptors in the isolated dog saphenous vein, mesenteric and renal arteries.

2.3.2.1.5 Therapeutic applications

Phenoxybenzamine is an irreversible antagonist of clinical importance. It was originally used for the treatment of essential hypertension, but was replaced with safer and more effective antihypertensive drugs. However, phenoxybenzamine is the drug of choice in the treatment of phaeochromocytoma (Hoffman, 2001). In addition, it has been proposed that phenoxybenzamine may be clinically employed to:

- inhibit the longitudinal smooth muscle contraction of the vas deferens and thereby act as a male contraceptive (Amobi & Smith, 1995),
- prevent postoperative vasospasm of radial artery conduits during and after radial artery grafts⁹ (Dipp *et al.*, 2001; Harrison *et al.*, 2001; Velez *et al.*, 2001),
- inhibit sympathetic neuronal-induced pain syndrome (CRPS)¹⁰ after peripheral nerve injuries (e.g. gunshot injuries in or near to the brachial plexus) and to treat severe cases of complex regional pain syndrome (Muizelaar *et al.*, 1997), and
- inhibit the contraction of the prostatic smooth muscle and to treat benign prostatic hyperplasia (Ruffolo & Hieble, 1999).

⁹ Radial artery conduits are prone to initial vasospasm induced by vasopressor therapy (to control blood pressure) intraoperatively and postoperatively, that may cause increased flow resistance in the grafts. Currently, intraoperative treatment (by soaking the conduits in a solution containing the non-specific antagonist papaverine) fails to provide sustained inhibition of vasoconstriction, and there is evidence that papaverine causes more endothelial damage than phenoxybenzamine (as confirmed by microscopic examinations).

¹⁰ Complex regional pain syndrome (CRPS) is classified as type I or II, with the known presence of a peripheral nerve injury in type II. Some criteria include spontaneous pain, blood circulation abnormalities of the skin and the absence of other conditions that would account for the degree of pain or dysfunction, e.g. diabetic neuropathy. CRPS has been formerly known as reflex sympathetic dystrophy or causalgia.

However, the notorious side effects of phenoxybenzamine may hamper these potential uses (see *Section 2.3.2.1.7*).

2.3.2.1.6 Pharmacokinetics

Phenoxybenzamine is presently available for clinical use as 10 mg dosage forms (Dibenzyl[®]) that can be administered intravenously or orally. The intravenous route is recommended to stabilise a patient suffering from a hypertensive crisis, due to the rapid onset of drug action and the complete bioavailability. Oral bioavailability is about 25%, the pharmacokinetic half-life approximately 24 hours and the metabolites of phenoxybenzamine are primarily excreted in the bile and urine (Moore, 1998). The pharmacological effects of phenoxybenzamine is, however, not a function of its plasma concentration due to its irreversible binding and long-lasting effect and the therapeutic (pharmacodynamic) half-life may be several days.

2.3.2.1.7 Toxicology

The major adverse affects of phenoxybenzamine include postural hypotension and sexual dysfunction in men (aspermia and inhibition of ejaculation). It has been reported that phenoxybenzamine has mutagenic activity in the Ames test and can therefore be regarded as potentially carcinogenic. The repeated administration of phenoxybenzamine in experimental animals caused peritoneal sarcomas and lung tumours (Hoffman, 2001). However, since its introduction into the clinical arena in 1953, no phenoxybenzamine-related tumours in humans have been reported after about 50 years of clinical experience with the drug (Te, 2002).

2.3.2.2 Benextramine

2.3.2.2.1 Chemical structure and properties

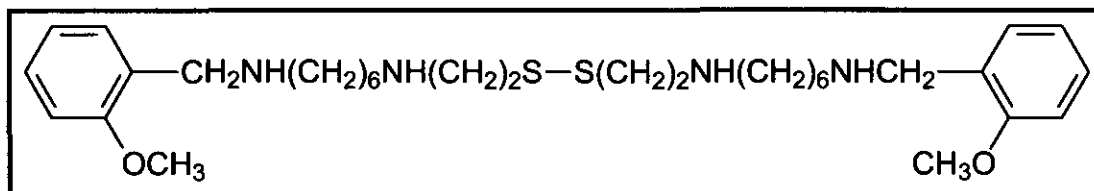


Figure 2–4: The chemical structure of benextramine. Note the centrally located disulphide (–S–S–) bridge that links two identical chemical groups.

In the early 1970s, a series of sulphur-containing polyamines was pharmacologically investigated in experimental animals, and these compounds were shown to interact selectively with the α -adrenoceptor (Demaree *et al.*, 1971; Herman *et al.*, 1971). Lippert & Belleau (1973) identified the chemical backbone of the active species and established that the inhibition of the α -adrenoceptor is irreversible and time-dependent. Consequently, the quantitative structure-activity relationships for a number of experimental compounds were determined, and a series with optimum activity at α -adrenoceptors identified, of which the prototype was benextramine (Benfey *et al.*, 1980; Melchiorre *et al.*, 1978 and Melchiorre, 1981).

2.3.2.2.2 Receptor interactions

It has been reported that benextramine binds irreversibly to 5HT_{1A}-serotonin receptors (Stanton & Beer, 1997), α -adrenoceptors (Melchiorre, 1981), H₂-histamine receptors (Belleau *et al.*, 1982), neuropeptide Y-receptors (Doughty *et al.*, 1990), and interacts non-specifically with prostanoid TP-receptors (Van der Graaf *et al.*, 1996).

2.3.2.2.3 Mechanism of action

The mechanism whereby benextramine inactivates α -adrenoceptors is different from the mechanism of receptor inactivation by phenoxybenzamine and 4-DAMP mustard. Melchiorre *et al.* (1979) investigated the role of the disulphide bridge of benextramine to inactivate the α -adrenoceptors in several isolated tissues of various species (e.g. rabbit aorta). They substituted the disulphide (–S–S–) bridge of the molecule with two

methylene groups and found that benextramine failed to inactivate the α -adrenoceptors under the same conditions. To explain their findings, they proposed that benextramine forms a covalent bond between the disulphide bridge of the molecule and a target thiol group in the active site of the α -adrenoceptor by means of a disulphide-thiol interchange reaction.

Observations made by Brasili *et al.* (1980) tend to support the proposal that a target thiol group may be involved, since they found that the covalent inhibition of α -adrenoceptors can be reversed by cationic thiols such as cysteamine. They conducted functional assays by measuring the effect of noradrenaline on the isolated rabbit aorta, and found that after pre-treatment with benextramine, cysteamine reverses the inhibition of contraction of the aorta in a concentration-dependent manner.

Additional studies by Melchiorre & Gallucci (1983) with benextramine on the α -adrenoceptors of the rat vas deferens demonstrate that two target thiol groups are probably involved in benextramine binding. By conducting protection experiments against benextramine blockade of the vas deferens with the haloalkylamine DMPEA and the classical calcium channel antagonist verapamil, their observations suggest that one of these thiol groups might possibly be located at the periphery of, or masked within, the calcium channel that is connected physiologically to the α -adrenoceptor.

It is also known that the amino acid cysteine contains a primary thioalcohol (thiol) group that is a nucleophile and may function as such during enzymatic catalysis. This amino acid is considered highly reactive. Therefore, benextramine binding probably involves cysteine residues in pharmacological receptors. Since the amino acid sequences of many receptors have been identified, structural models of these receptors with the positions of cysteine residues might give possible indications of the binding sites involved with benextramine. Important for this study is the presence of three cysteines in the extracellular parts of the porcine α_{2A} -adrenoceptor (one each in the extracellular loops e1, e2 and e3) (Guyer *et al.*, 1990), four in the extracellular parts of the human M_3 muscarinic acetylcholine receptor (one each in e1 and e2, and two in e3) (Peralta *et al.* 1987), and eight in the extracellular parts of the human 5HT $_{2A}$ -serotonin receptor (four in the N-terminal extension, one each in e1 and e2, and two in e3) (Saltzman *et al.*, 1991).

In addition, the irreversible binding of benextramine to cysteine residues on receptors modifies the properties of the sulphydryl groups, that have been shown to play important roles in the coupling of receptors to G proteins and the functioning of the receptor-G protein interfaces (Kennedy & Limbird, 1983). It has also been shown that α_{2A} -adrenoceptors preferentially interact with G_i rather than G_s or G_q proteins (Chabre *et al.*, 1994). Many studies have been conducted to understand the structural determinants of G protein coupling and its activation by GPCRs. It has been proposed that specific regions of α_{2A} -adrenoceptors are responsible for the activation of G proteins, of which i1 and i3 have been shown to be most critical (Dalman & Neubig, 1991; König *et al.*, 1989). Eason & Liggett (1996) and Wade *et al.* (1996) showed the importance of i3 to serve as a G_i activator domain for the α_{2A} -adrenoceptor.

2.3.2.2.4 Experimental applications

Benextramine is one of the classical irreversible α -adrenoceptor antagonists that have been experimentally employed in numerous published studies. For example, Taouis *et al.* (1986) examined the turnover of α_{2A} -adrenoceptors in the adipose and kidney tissues of golden hamsters, and McKernan *et al.* (1988) employed benextramine to inactivate α_2 -adrenoceptors on the surface of human erythroleukemia cells to investigate receptor compartmentation.

2.3.2.2.5 Therapeutic applications

Currently, benextramine has not been employed therapeutically.

2.3.2.3 4-DAMP mustard

2.3.2.3.1 Chemical structure and properties

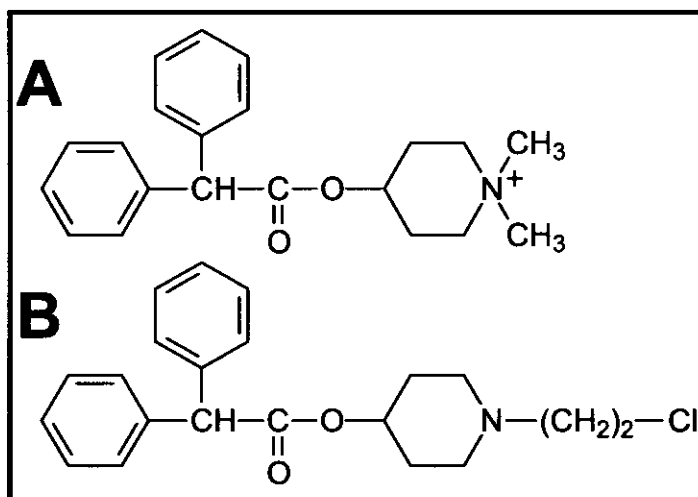


Figure 2–5: The chemical structures of **(A)** 4-DAMP and **(B)** 4-DAMP mustard. Note the structural similarities between the two compounds. 4-DAMP contains two methyl groups on the nitrogen atom and 4-DAMP mustard a chloroethylene chain.

4-DAMP mustard is the 2-chloroethylamine derivative of the competitive muscarinic acetylcholine receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) (Thomas *et al.*, 1992), shown to be selective for M₂-receptors (Barlow & Shepherd, 1986). *Figure 2–5* shows that the two methyl groups on the nitrogen atom of 4-DAMP (*Figure 2–5A*) were substituted with a chloroethylene group to form the haloalkylamine 4-DAMP mustard (*Figure 2–5B*).

2.3.2.3.2 Receptor interactions

It has been reported that 4-DAMP mustard interacts irreversibly with muscarinic acetylcholine receptors (i.e. M₁ to M₅) and does not discriminate between the subtypes of these receptors (Eglen *et al.*, 1994; Ehlert, 1996; Thomas *et al.*, 1992).

Thomas *et al.* (1992) investigated the conversion of 4-DAMP mustard to its aziridinium ion and the interactions with muscarinic acetylcholine receptors in various tissues (e.g. the isolated rat submaxillary gland and left atrium). They observed that when 4-DAMP mustard is dissolved in an aqueous solution at pH 7.4 and 37°C, the aziridinium ion reached a peak concentration after 32 minutes, corresponding to about

75% of the initial concentration of 4-DAMP mustard. They also determined the affinity of 4-DAMP mustard and its transformation products for muscarinic acetylcholine receptors by conducting competition binding assays. Their results show that the aziridinium ion of 4-DAMP mustard is more potent than the parent 2-chloroethylamine. In addition, the aziridinium ion of 4-DAMP mustard exhibits slight selectivity for M₃-muscarinic acetylcholine receptors in the isolated rat submaxillary gland, compared with M₂-receptors in the isolated rat atrium.

2.3.2.3.3 Experimental applications

4-DAMP mustard is one of the classical irreversible muscarinic acetylcholine receptor antagonists that have been experimentally employed in numerous published studies. The property of 4-DAMP mustard not to discriminate between muscarinic acetylcholine receptor subtypes proved to be useful and resulted in the drug being employed extensively. For instance, a selective reversible antagonist is used prior to treatment with 4-DAMP mustard to protect a specific muscarinic acetylcholine receptor subtype. Thereafter, the reversible competitive antagonist is rinsed from the pharmacological system and the function of the remaining (protected) muscarinic acetylcholine receptors investigated, e.g. by measuring an agonist-induced pharmacological effect. For example, Braverman & Ruggieri (1999) employed 4-DAMP mustard to inactivate M₃-receptors and selectively investigate the acetylcholine-induced contractile function of M₂-receptors in the isolated smooth muscle of the rat urinary bladder. Similarly, Yamanishi *et al.* (2002) investigated the function of the acetylcholine-induced M₂-receptor-mediated contraction of the isolated smooth muscle of the pig urinary bladder base after selective inactivation of M₃-receptors with 4-DAMP mustard.

2.3.2.3.4 Therapeutic applications

Currently, 4-DAMP mustard has not been employed therapeutically.

2.3.3 Evidence for irreversible non-specific antagonism

As mentioned in *Section 2.2.4*, it is generally assumed that irreversible antagonists bind irreversibly to syntopic binding sites on pharmacological receptors and that they thereby prevent agonists (and other ligands) from binding to the receptor. Since the agonist can no longer elicit a pharmacological effect at the antagonist-bound receptors, these receptors are considered inactivated. However, the possibility, probability and concerns that irreversible antagonists may also interact with non-syntopic binding sites on the receptor or even on other proteins in the signalling pathway, have been raised. The following observations and reasoning that support the concerns will be discussed in more detail in this section:

- Most irreversible antagonists are known to be chemically highly reactive. They bind covalently with many entities and may, besides binding to syntopic binding sites on receptors, be likely to also bind non-syntopic binding sites (Bodenstein, 2000; Brink, 1997).
- Most irreversible antagonists do not inactivate selective receptor types or even discriminate between receptor subtypes. For example, it has been observed that the irreversible antagonist phenoxybenzamine inhibits agonist-induced effects through many receptor types and subtypes (see *Section 2.3.2.1.2*).

In addition, the possibility that irreversible antagonists may interact with non-specific receptors has not been studied extensively, despite only a few suggestions in support thereof in literature outlined below.

2.3.3.1 Dibenamine and agonist binding kinetics at muscarinic acetylcholine receptors

The primary concerns that an irreversible antagonist may interact with non-syntopic binding sites on pharmacological receptors appeared from reports by Van Ginneken (1977). He investigated the antagonism of the irreversible antagonist dibenamine (structurally and chemically related to phenoxybenzamine) by measuring the effects of the muscarinic acetylcholine receptor agonist methylfurfurethonium on the isolated guinea-pig ileum. His studies consisted of kinetic assays where he measured the effect

(contraction) obtained with various concentrations methylfurethronium. By comparing the results obtained with and without dibenamine pre-treatment, he observed that although pre-treatment with dibenamine reduced the maximal effect to about 10% of the control, the rate at which this maximal effect was attained, increased. *Figure 2-6* depicts the results obtained with the methylfurethronium-induced contraction rate of the isolated guinea-pig ileum before and after pre-treatment with dibenamine (Van Ginneken, 1977).

To explain his observations, Van Ginneken (1977) reasoned that pre-treatment with dibenamine not only resulted in the inactivation of a fraction of muscarinic acetylcholine receptors and a consequent decrease in the methylfurethronium-induced effect, but dibenamine possibly modulates the rate constants involved in the binding of methylfurethronium to the remaining fraction of operative receptors. He proposed that dibenamine (and probably many other irreversible antagonists) acts non-specifically by inducing conformational changes in the functional receptors to change ligand-receptor binding kinetics.

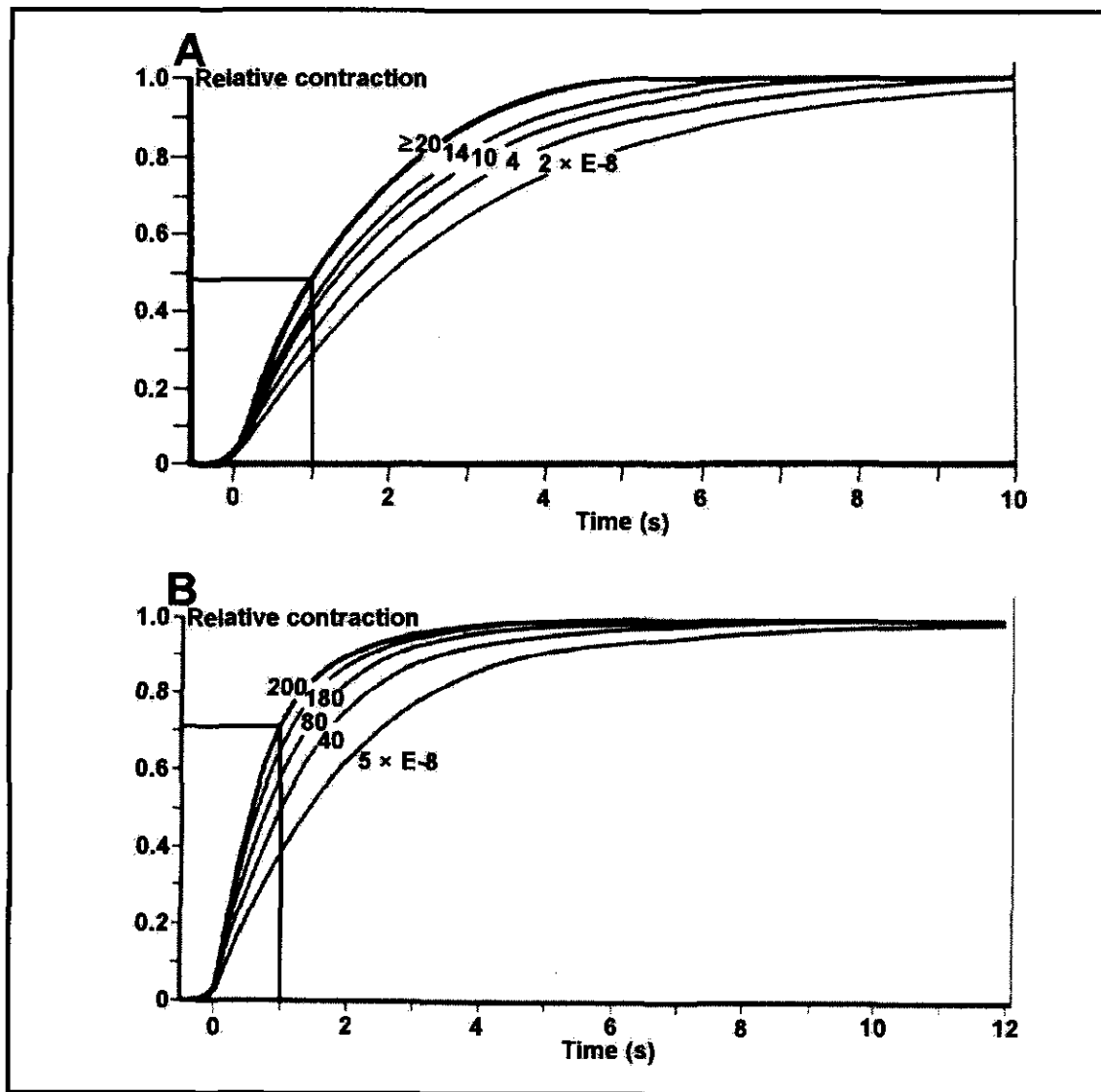


Figure 2-6: Relative isometric contractions of the isolated guinea-pig ileum as a function of time with various concentrations (given in molar) of the muscarinic acetylcholine receptor agonist methylurethronium. The effect was measured **(A)** without pre-treatment with the irreversible antagonist dibenamine, and **(B)** after pre-treatment with dibenamine. When comparing **(A)** with **(B)**, it is evident that pre-treatment with dibenamine in **(B)** increased the rate at which the maximal effect was obtained. For example, the relative maximal effect obtained with the highest concentration methylurethronium after 1 s in **(A)** was ~50%, compared to ~70% in **(B)**. Note that dibenamine pre-treatment decreased the effect in **(B)** to ~10% of the control (this is not evident in the figure, since relative effects for each condition were measured). Figure adapted from Van Ginneken (1977).

2.3.3.2 Benextramine acts non-specifically at prostanoid TP-receptors

Van der Graaf *et al.* (1996) studied the mechanism of action of the irreversible antagonist benextramine by measuring the effect of the prostanoid TP-receptor agonist U46619 on the isolated rat small mesenteric artery. They conducted functional assays

by measuring the contraction of the artery obtained with various concentrations U46619 after pre-treatment with the drug vehicle, benextramine alone, and with benextramine either in the presence of a relative high concentration of the prostanoid TP-receptor antagonist SQ 30,741, or a relative high concentration of U46619. By comparing the results at each condition, they observed that neither SQ 30,741, nor U46619 could protect against the irreversible antagonism of benextramine. *Figure 2–7* depicts the results obtained for each condition by measuring the contraction of the artery as a function of the log concentration U46619 (Van der Graaf *et al.*, 1996).

Van der Graaf *et al.* (1996) reasoned that if benextramine inhibits the effect of U46619 by inactivating specific receptors on the prostanoid TP-receptor macromolecule, then SQ 30,741 should be able to prevent the observed antagonism by benextramine. However, they observed that neither SQ 30,741 (at a concentration of ~250 times the apparent affinity for prostanoid TP-receptors in the isolated rat small mesenteric artery), nor U46619 (at a concentration that results in a near-maximal effect) could produce significant protection against the antagonistic effects of benextramine. To explain their observations, they reasoned that benextramine does not bind to the same receptor site as U46619 and SQ 30,741, and concluded that benextramine acts as an irreversible non-specific antagonist at prostanoid TP-receptors. They did not further investigate the nature of the observed non-specific antagonism.

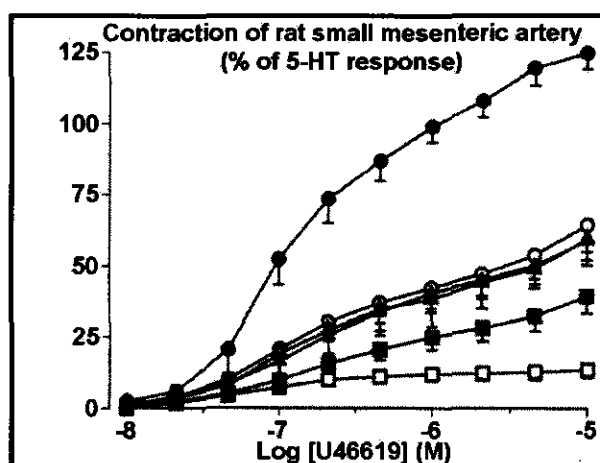


Figure 2-7: Semilogarithmic concentration-effect curves of the selective prostanoid TP-receptor agonist U46619 on the isolated rat small mesenteric artery (% of contraction obtained with 30 μ M serotonin). Curves were constructed following pre-treatment with the drug vehicle ($\bullet$), 100 μ M benextramine alone for 30 ($\circ$), 60 ($\blacksquare$) and 120 minutes ($\square$), or 100 μ M benextramine for 30 minutes in the presence of either 10 μ M of the selective prostanoid TP-receptor antagonist SQ 30,741 ($\blacktriangle$) or 10 μ M ($\triangle$) U46619. Note that irreversible inhibition cannot be surmounted with relative high concentrations of either U46619, or SQ 30,741 (as indicated by a persistent reduction in the maximal effect, when compared to the control). Figure adapted from Van der Graaf *et al.* (1996).

2.3.3.3 Benextramine may act non-specifically at α_{2A} -adrenoceptors

Brink *et al.* (2000) utilised the familiar Furchgott analysis (see above) in functional assays by employing benextramine to inactivate porcine α_{2A} -adrenoceptors in transfected Chinese hamster ovary cells. They employed the full α_{2A} -adrenoceptor agonist UK 14,304 and obtained estimated apparent pK_A values of UK 14,304 at α_{2A} -adrenoceptors for the G_s and G_i signalling pathways, after partial receptor alkylation by increasing concentrations benextramine. These values were employed to determine estimates of the relative efficacies for a series of full α_{2A} -adrenoceptor agonists. In addition, they determined the K_i value of UK 14,304 at α_{2A} -adrenoceptors for the G_s and G_i signalling pathways from competition binding assays.

By comparing the results of the estimated K_i value with the estimated apparent K_A values (as estimated from the Furchgott analysis of the concentration-effect curves after pre-treatment with 1, 10 or 100 μ M benextramine), they found the K_A value with 100 μ M benextramine to be between three- and five-fold higher than the K_i value, for the G_s and G_i signalling pathways respectively. Table 2-1 depicts the apparent pK_A values and ratios of apparent K_A value to K_i value obtained for UK 14,304 at different

signalling pathways as a function of the benextramine concentration (Brink *et al.*, 2000). They concluded that more reliable K_A values may be calculated at the lowest concentration benextramine, since non-specific antagonism may be involved at the highest concentration.

Table 2–1: The apparent pK_A values and ratios of $K_A(\text{apparent}):K_i$ of UK 14,304 at porcine α_{2A} -adrenoceptors for the G_s and G_i signalling pathways in transfected Chinese hamster ovary cells. The $K_A(\text{app.})$ values were calculated from the Furchgott analysis of concentration-effect curves after partial receptor alkylation with increasing concentrations benextramine. Table adapted from Brink *et al.* (2000).

[Benextramine] (μM)	G_s Pathway		G_i Pathway	
	$pK_A(\text{app.})$	$K_A(\text{app.})/K_i$	$pK_A(\text{app.})$	$K_A(\text{app.})/K_i$
1	6.39	0.85	6.23	1.22
10	6.31	1.02	6.33	0.99
100	5.83	3.08	5.62	5.0

2.3.3.4 Dibenamine and phenoxybenzamine change the Hill slope of concentration-effect curves

Brink (1997) and Bodenstein (2000) analysed the concentration-effect curves of data obtained from literature and from experiments conducted on various isolated animal organs (e.g. rat aorta, jejunum and vas deferens; guinea-pig tracheal chain). They noticed that, after pre-treatment with the irreversible antagonists dibenamine and phenoxybenzamine, the Hill slopes of the concentration-effect curves deviated significantly from unity, and these irreversible antagonists probably modulate signal transduction by altering the receptor-effector coupling. A Hill slope that deviates from unity could mean a non-linear and variable relationship between the fraction of receptors occupied (stimulus) and one or more transductional events that follow to elicit a pharmacological effect (Jenkinson, 2003). For example, it is likely that the irreversible antagonist binds to non-synaptic binding sites and thereby alters the affinity for the agonist (a phenomenon referred to as negative cooperativity) (Gibb, 2003).

2.4 Conclusionary remarks

Irreversible antagonists such as phenoxybenzamine, benextramine and 4-DAMP mustard are drugs that bind irreversibly to various binding sites on various types of pharmacological receptor macromolecules. Thereby they inhibit the binding of other ligands such as agonists to these binding sites. By inactivating syntopic binding sites, irreversible antagonists decrease the number of syntopic binding sites available for binding to an agonist. This results in a decrease in the stimulus induced, and in the absence of spare receptors, a reduction in the maximal pharmacological effect. However, there are also suggestions that irreversible antagonists may bind irreversibly to non-syntopic binding sites on the same receptor macromolecule that the agonist binds to, or may even involve binding to a molecular locus distinct from the receptor, and thereby modulate the relationship between stimulus and pharmacological effect. This could influence the interpretation of data obtained after pre-treatment with an irreversible antagonist.

Irreversible antagonists have been extensively employed in experimental pharmacology. Phenoxybenzamine is also clinically employed. Important experimental applications for irreversible antagonists include investigations into the non-linear stimulus-effect relationships for agonists in various pharmacological systems, the types of binding sites on receptor macromolecules, or the pharmacological function of selected receptors.

The classical Furchgott analysis has been one important approach to employ irreversible antagonists to determine estimates of the equilibrium dissociation constants of agonist-receptor complexes and the relative intrinsic efficacies of agonists at their specific receptors, and thereby to classify agonists. However, it is assumed that the irreversible antagonist employed in the Furchgott analysis binds only to syntopic binding sites and thereby decreases the receptor number available for an agonist, and the drug should not bind to non-syntopic binding sites that modulate the subsequent stimulus-effect relationship or the affinity of the receptors for the agonist. Concerns have been raised that some irreversible antagonists may also bind to non-syntopic binding sites and thereby modulate signalling within the remaining fraction of functional receptors, or the affinity of the receptors for an agonist. Such mechanisms would render the Furchgott analysis invalid and may prompt for the re-evaluation of

data obtained with such irreversible antagonists. These concerns need further investigation, as in the present study where a selection of classical irreversible antagonists has been chosen to investigate any potential non-specific mechanisms of antagonism at the conditions applied.

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***The Classical Irreversible
Competitive Antagonists
Phenoxybenzamine,
Benextramine and 4-DAMP
Mustard Display Non-
competitive Antagonism***

**Chapter
3**

Full Paper

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Summary

1. Irreversible antagonists have been used to study agonists and spare receptors by implementing the Furchgott analysis. This analysis assumes that equal agonist effect before and after treatment with the irreversible antagonist represents equal stimulus. However, when irreversible antagonists display non-specific mechanisms of antagonism, this analysis is invalid. Non-specific mechanisms of antagonism have not been thoroughly investigated for classical irreversible antagonists.
2. We have studied the non-specific mechanisms in the irreversible antagonism of phenoxybenzamine, benextramine on α_{2A} -adrenoceptors in transfected Chinese hamster ovary cells and of 4-DAMP mustard on muscarinic acetylcholine (mACh) receptors in human neuroblastoma SH-SY5Y cells.
3. α_{2A} -Adrenoceptors and mACh receptors were protected by appropriate reversible competitive antagonists (0 or 10 μ M yohimbine or atropine, respectively) and then pre-treated with phenoxybenzamine (1, 10 or 100 μ M for 20 minutes), benextramine (1, 10 or 100 μ M for 20 minutes; or 10 μ M for 60 and 120 minutes) or 4-DAMP mustard (10 or 100 nM for 20 minutes). Thereafter, receptor concentrations were measured by radioligand binding studies, concentration-effect curves of appropriate agonists constructed and agonist affinity measured by competition binding studies.
4. Phenoxybenzamine (100 μ M; 20 minutes), benextramine (10 μ M or 100 μ M; 20 minutes) and 4-DAMP mustard (100 nM; 120 minutes) displayed irreversible non-specific (metastoid) antagonism. Longer incubation times greatly enhanced non-specific antagonism.
5. We conclude that irreversible antagonists should be evaluated appropriately for non-specific antagonism under experimental conditions used, before application in experiments where this may influence the interpretation of results.

Keywords

Irreversible competitive antagonist; relative intrinsic efficacy; non-specific; Furchgott analysis; spare receptors; α_{2A} -adrenoceptors; muscarinic acetylcholine receptors; benextramine; phenoxybenzamine; 4-DAMP mustard

Abbreviations

4-DAMP mustard, 4-diphenylacetoxy-*N*-[2-chloroethyl]piperidine hydrochloride; α_{2A} -AH, α_{2A} -adrenoceptors expressed at relative high numbers; α_{2A} -AL, α_{2A} -adrenoceptors expressed at relative low numbers; ATP, adenosine triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; CHO, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; E_{max} , maximum effect; EMEM, minimum essential medium (Earle's base); HEPES, *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulphonic acid]; IBMX, 3-isobutyl-1-methylxanthine; IP_x , total inositol phosphates; mACh receptor, muscarinic acetylcholine receptor; methacholine, acetyl- β -methylcholine chloride; PBS, phosphate buffered saline; SH-SY5Y, cultured human neuroblastoma cell line; TCA, trichloroacetic acid; UK 14,304, 5-bromo-*N*-[2-imidazolin-2-yl]-6-quinoxalinamine (brimonidine); UltraMEM, reduced serum minimum essential medium

3.1 Introduction

Irreversible competitive antagonists (e.g. β -haloalkylamines) are generally accepted to bind pharmacological receptors irreversibly (usually by alkylation) and thereby preventing other ligands from binding to these receptors. The classical irreversible α -adrenoceptor blocking drug phenoxybenzamine has been used for many years in the clinical setting to treat phaeochromocytoma (Hoffman, 2001). Most irreversible competitive antagonists, however, found application in experimental pharmacology where they have been employed to eliminate spare receptors (partial receptor elimination) to investigate the phenomenon of receptor reserve, or, by implementing the Furchgott analysis (Furchgott, 1966), to estimate the relative intrinsic efficacy of agonists and the apparent equilibrium dissociation constants ($K_A(\text{app.})$ value) of agonist-receptor complexes (Adham *et al.*, 1993; Agneter *et al.*, 1993; Herepath & Broadley, 1990; Kenakin, 1997; Koek *et al.*, 2000; Morey *et al.*, 1998; Tian *et al.*, 1996; Zhu, 1993). Irreversible antagonists have also been implemented to label and count receptor subtypes, to investigate drug and receptor specificity and receptor structure (Jenkinson, 2003) and to unravel drug action mechanisms (Timmermans *et al.*, 1985). Selective alkylation of receptors has also been implemented as a pharmacological tool to eliminate unwanted receptor subtypes and then to study and characterise the pharmacological effects of the remaining receptor subtype of interest, where these subtypes were protected from inactivation by the irreversible competitive antagonist by employing an appropriate concentration of a highly selective reversible competitive antagonist (Eglen *et al.*, 1994; Hieble *et al.*, 1985).

There are several irreversible competitive antagonists known and used *in vitro* in experimental pharmacology. Examples that are important for this study include **phenoxybenzamine**, binding to α -adrenergic, H_1 -histamine and muscarinic acetylcholine (mACh) receptors (Amobi & Smith, 1995; Eglen *et al.*, 1994; Frang *et al.*, 2001; Furchgott, 1966; Giardinà *et al.*, 1995; Giardinà *et al.*, 2002; Ruffolo & Hieble, 1999; Van der Graaf & Danhof, 1997; Van der Graaf & Stam, 1999), **benextramine**, binding selectively to α_2 -adrenoceptors (Belleau *et al.*, 1982; Brink *et al.*, 2000; Hieble *et al.*, 1985; Lew & Angus, 1984; McKernan *et al.*, 1988; Melchiorre, 1981; Taouis *et al.*, 1986; Timmermans *et al.*, 1985; Umland *et al.*, 2001), and 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine (**4-DAMP mustard**), binding to mACh

receptors (Eglen *et al.*, 1994; Ehlert & Griffin, 1998; Ragheb *et al.*, 2001; Sawyer & Ehlert, 1999; Thomas *et al.*, 1992; Umland *et al.*, 2001). 4-DAMP mustard demonstrates moderate selectivity for M₃-mACh receptors, but does not discriminate between M₁, M₂ or M₄-mACh receptors (Eglen *et al.*, 1994). Other examples, illustrating the extensive use of irreversible competitive antagonists *in vitro* in experimental pharmacology, include the classical α -adrenoceptor selective antagonist dibenamine (Siegl & McNeill, 1982), the α_{1B} -adrenoceptor selective antagonist chloroethylclonidine (CEC) (Giardinà *et al.*, 1995), the α_{2D} -adrenoceptor selective antagonist *N*-ethoxycarbonyl-1,2-dihydroquinoline (Tian *et al.*, 1996), the β -adrenoceptor selective antagonist Ro 3-7894 (Siegl & McNeill, 1982), the A₁-adenosine receptor selective antagonist FSCPX (8-cyclopentyl-3-[3-[[4-(fluorosulphonyl)benzoyl]oxy]propyl]-1-propylxanthine) (Morey *et al.*, 1998), the mACh receptor and 5HT_{1B}-serotonin receptor binding antagonist *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Adham *et al.*, 1993; Agneter *et al.*, 1993; Koek *et al.*, 2000) and the mACh receptor selective antagonist propylbenzilylcholine mustard (Eglen *et al.*, 1994).

The number of receptors that are eliminated by treatment with an irreversible competitive antagonist is dependent on both the concentration used and the incubation time (Furchgott, 1966; Kenakin, 1997). In this regard and of interest for the present study, phenoxybenzamine has been used *in vitro* at concentrations of up to 10 μ M for 30 minutes incubation time (Piascik *et al.*, 1988), while benextramine has been used at concentrations of up to 100 μ M for 120 minutes incubation time (Van der Graaf *et al.*, 1996) and 4-DAMP mustard at concentrations of up to 40 nM for 4 hours incubation time (Sawyer & Ehlert, 1999).

As mentioned previously, the Furchgott analysis has been implemented to investigate agonist efficacy and apparent affinity after elimination of spare receptors by irreversible antagonists. This analysis is primarily based on the assumption that equal submaximal effects of an agonist before and after treatment of the receptors with the irreversible competitive antagonist result from equal stimuli. The comparison of equal submaximal effects is therefore assumed to represent conditions of equal receptor occupancy by the agonist and it is assumed that no change has occurred with respect to the stimulus-effect relationship (Furchgott, 1966; Kenakin, 1997). It is therefore

essential that the irreversible competitive antagonist selectively binds to the receptor binding site (inactivating the bound receptors) and that it does not interfere non-specifically with the signal transduction of the remaining operative receptors (Jenkinson, 2003).

However, reports in literature suggest that the irreversible competitive antagonists may also display irreversible non-competitive (non-specific) mechanisms of antagonism (Brink *et al.*, 2000; Van der Graaf *et al.*, 1996), or that these antagonists may modify agonist-receptor binding kinetics (Van Ginneken, 1977), although this has been difficult to prove (Jenkinson, 2003). This implies that the irreversible competitive antagonists may also interact with non-specific (non-synaptic) binding sites and thereby influence the signal transduction pathway or the binding properties of the receptor to other ligands. Such mechanisms are incompatible with the basic assumptions inherent to the Furchgott analysis, so that in these cases the analysis cannot be applied to determine estimates of the equilibrium dissociation constants of agonist-receptor complexes or the relative intrinsic efficacies of agonists.

The first substantial evidence indicating that benextramine displays irreversible non-specific antagonism at prostanoid TP-receptors in the rat small mesenteric artery was reported by Van der Graaf *et al.* (1996). Relatively high concentrations of the selective prostanoid TP-receptor antagonist SQ 30,741 were employed to protect these receptors against the inactivation by benextramine. An important finding was that the antagonist could not protect against the irreversible antagonistic effects of benextramine. These data suggest that benextramine and SQ 30,741 do not bind to the same receptor binding site, but the underlying mechanism of the non-specific antagonism by benextramine remained a mystery.

In the present study it was investigated whether three classical irreversible competitive antagonists, namely benextramine and phenoxybenzamine at α_{2A} -adrenoceptors and 4-DAMP mustard at mACh receptors, display non-specific mechanisms of irreversible antagonism (in addition to their known specific mechanisms), and if so, whether the mechanism is metaaffinoid (allosteric, modifying agonist affinity) or metactoid (functional, modifying signal transduction) in nature.

3.2 Methods

3.2.1 Cell lines

In this study cultured cell lines expressing α -adrenoceptors and muscarinic acetylcholine (mACh) receptors, respectively, were employed. Two Chinese hamster ovary (CHO-K1) cell lines, transfected to express the wild-type porcine α_{2A} -adrenoceptor at high numbers (cell line denoted α_{2A} -H) and low numbers (cell line denoted α_{2A} -L) respectively, were a kind gift from Dr Rick Neubig, Departments of Pharmacology and Internal Medicine (Division of Hypertension), University of Michigan, Ann Arbor, MI, U.S.A. The pharmacological profiles and receptor expression characteristics of the α_{2A} -H and α_{2A} -L cell lines have been previously characterised. The determined α_{2A} -adrenoceptor concentrations were reported as 19 ± 2 pmol mg⁻¹ membrane protein for α_{2A} -H and as about 1 pmol mg⁻¹ membrane protein for α_{2A} -L (Brink *et al.*, 2000). These cell lines were used to investigate the mechanisms of antagonism of the irreversible α -adrenoceptor blocking drugs phenoxybenzamine and benextramine. We also used human neuroblastoma cells (SH-SY5Y, from American Type Culture Collection), that endogenously express predominantly M₃-mACh receptors (Słowiejko *et al.*, 1996), with some evidence for M₁ and M₂-mACh receptors (Kukkonen *et al.*, 1992). The latter cell line was employed to evaluate mechanisms of antagonism of the irreversible mACh receptor blocking drug 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine (4-DAMP mustard).

The α_{2A} -L and α_{2A} -H cells were maintained and grown to 95% confluency in 150 cm² cell culture flasks with Ham's F-12 medium with 10% fetal bovine serum, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C in 5% CO₂. The SH-SY5Y cells were also maintained and grown to 95% confluency in 150 cm² cell culture flasks, but with a 1:1 ratio mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) with 10% bovine serum albumin, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C in 5% CO₂.

3.2.2 Preparation and pre-treatment with irreversible competitive antagonists

Cells were seeded in 24-well plates in preparation for the [^3H]-adenosine 3',5'-cyclic monophosphate ([^3H]-cAMP), [^3H]-total inositol phosphates ([^3H]-IP_x) or ligand binding assays, as described below, and maintained for at least 18 hours at 37°C in 5% CO₂. Cells attached adequately to the well bottoms, allowing several aspirations and new additions of medium without significant cell loss (confirmed by microscopic observation).

When the experiments were performed the pre-treatments were initiated by incubating the cells with an appropriate concentration (0 M or a concentration $> 1000 \times K_D$ value) of the reversible antagonist (allowing equilibrium of ligand-receptor binding), whereafter the cells were exposed to different concentrations of the appropriate irreversible antagonist plus the reversible antagonist. This was followed by several rinsing and incubation steps (washing procedure) with phosphate buffered saline (PBS; containing 0.8% NaCl, 0.02% KCl, 0.09% Na₂HPO₄ and 0.02% KH₂PO₄) and DMEM to remove all unbound and reversibly bound drugs. The following pre-treatment steps were employed: (1) α_{2A} -L or α_{2A} -H cells were incubated with either 0 or 10 μM of the reversible competitive α_2 -adrenoceptor antagonist yohimbine hydrochloride (Becker *et al.*, 1999) in DMEM for 30 minutes at 37°C in 5% CO₂, to allow equilibrium of ligand-receptor binding. Likewise, SH-SY5Y cells were incubated with either 0 or 10 μM of the reversible competitive non-selective mACh receptor antagonist atropine sulphate (Zwart & Vijverberg, 1997) in DMEM for 30 minutes at 37°C in 5% CO₂, to allow equilibrium of receptor binding. (2) Thereafter α_{2A} -L or α_{2A} -H cells were correspondingly incubated with either 0 or 10 μM of yohimbine plus the indicated concentration of phenoxybenzamine hydrochloride or benextramine tetrahydrochloride (0, 1, 10 or 100 μM) for 20 minutes (or 60 or 120 minutes when indicated) at 37°C in 5% CO₂.

SH-SY5Y cells were correspondingly incubated with either 0 or 10 μM of atropine plus the indicated concentration of 4-DAMP mustard (0, 10 or 100 nM) for 20 minutes (or 120 minutes when indicated) at 37°C in 5% CO₂. All solutions were freshly prepared immediately before each assay was conducted. Since 4-DAMP mustard is

completely converted to its corresponding active aziridinium ion only after about 30 minutes in aqueous solution (Thomas *et al.*, 1992), it was kept for at least 30 minutes at 37°C in DMEM before use in the assay. (3) Cells were then rinsed twice with PBS and incubated twice with pure DMEM for 20 minutes to allow dissociation of any reversibly bound drugs, whereafter the cells were used for the functional or ligand-binding assays as described below.

3.2.3 Measurement of whole-cell [^3H]-cAMP accumulation

[^3H]-cAMP accumulation was determined in whole α_{2A} -L cells in 24-well plates as described by Wade *et al.* (1999) and Wong (1994). Briefly, cells were labelled by adding 1 μCi per well [$2\text{-}^3\text{H}$]-adenine at least 18 hours before the assay. After the washing procedure (as described above), the assay was initiated by adding DMEM with 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 30 μM forskolin and the appropriate concentration of the full α_{2A} -adrenoceptor agonist UK 14,304 (Brink *et al.*, 2000) to construct appropriate semilogarithmic concentration-effect curves. After a 20 minute incubation time at 37°C in 5% CO_2 , the medium was aspirated and the reaction terminated with 1 ml ice-cold 5% trichloroacetic acid (TCA) containing 1 mM adenosine triphosphate (ATP) and 1 mM cAMP and allowed to stand for 30 minutes at 4°C to lyse the cells. The acid soluble nucleotides were separated on Dowex and alumina columns as described by Salomon *et al.* (1974) and radioactivity determined by liquid scintillation counting. The cAMP accumulation was normalised by dividing the [^3H]-cAMP counts by the total [^3H]-nucleotide counts. This value was then divided by the corresponding value obtained in the presence of IBMX and forskolin, without drug (to calculate percentage of control).

3.2.4 Measurement of whole-cell [^3H]-IP_x accumulation

[^3H]-IP_x accumulation was determined in whole SH-SY5Y cells in 24-well plates according to the principles described by Godfrey (1992) and the procedure was followed essentially as described by Casarosa *et al.* (2001), but with minor modifications. Briefly, cells were labelled by adding 1 μCi per well *myo*-[$2\text{-}^3\text{H}$]-

inositol in inositol-free medium (EMEM, Earle's base + bovine serum albumin) at least 18 hours before the assay. After the pre-treatment and washing procedure (as described above), the [^3H]-IP_x assay was initiated by adding a mixture of DMEM, 20 mM LiCl, 25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) and the appropriate concentration of the full mACh receptor agonist methacholine chloride (Olianas & Onali, 1991) to construct appropriate semilogarithmic concentration-effect curves. After a 60-minute incubation at 37°C and 5% CO₂ the medium was aspirated and the reaction terminated with 1 ml ice-cold 10 mM formic acid and let to stand for at least 90 minutes at 4°C to lyse the cells. The [^3H]-IP_x was separated on Dowex columns (250 μl Dowex 1 $\times$ 8-400, 200-400 mesh, 1-chloride form per 2 ml, Bio-Rad Poly-Prep column) and radioactivity determined by liquid scintillation counting. The [^3H]-IP_x accumulation was expressed as the percentage of the control value measured in the absence of agonist.

3.2.5 Ligand binding assays

The K_D value of the α_{2A} -adrenoceptor competitive antagonist yohimbine was determined from radioligand saturation binding experiments in whole α_{2A} -H cells. Cells were plated and incubated for at least 18 hours at 37°C in 5% CO₂ as before, but without a radioligand. The cells were then rinsed once with reduced serum minimum essential medium (UltraMEM), whereafter the assay was initiated by adding UltraMEM with 0 or 10 μM yohimbine (to define non-specific binding) plus the appropriate concentration of [*O*-methyl- ^3H]-yohimbine. After a 30 minute incubation at 37°C in 5% CO₂, the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml ice-cold 5% TCA and let to stand for at least 30 minutes at 4°C to lyse the cells. The TCA from each well was then transferred directly into scintillation vials and the radioactivity (bound [*O*-methyl- ^3H]-yohimbine) counted.

After the pre-treatments described above, the pK_i value of the α_{2A} -adrenoceptor agonist UK 14,304 was determined from competition binding curves in whole α_{2A} -H cells against 5 nM [*O*-methyl- ^3H]-yohimbine ($K_D = 3.67 \pm 0.00$ nM). In preparation for the competition binding experiments the cells were plated and incubated as before, but without a radioligand. Cells were then rinsed once with UltraMEM, whereafter the assay was initiated by adding UltraMEM with 5 nM [*O*-methyl- ^3H]-yohimbine and

different concentrations of UK 14,304. After a 30 minute incubation at 37°C in 5% CO₂, the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml of 5% TCA and let to stand for at least 30 minutes to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials and the radioactivity (bound [*O*-methyl-³H]-yohimbine) counted.

The relative α_{2A} -adrenoceptor concentrations in α_{2A} -H cells (after the various pre-treatments with the appropriate irreversible competitive antagonists and washing procedure as described above) were determined from specific binding of 5 nM [*O*-methyl-³H]-yohimbine (adding also 0 M yohimbine, or 10 μ M yohimbine to define non-specific binding). Likewise, the mACh receptor relative concentrations in SH-SY5Y cells were determined from specific binding by 5 nM [*N*-methyl-³H]-4-DAMP (adding 0 M atropine, or 10 μ M atropine to define non-specific binding). In all cases, after a 30 minute incubation at 37°C in 5% CO₂, the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml of 5% TCA and let to stand for at least 30 minutes to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials and the radioactivity (bound [*O*-methyl-³H]-yohimbine) counted.

3.2.6 Data analysis

Data from all studies were obtained from triplicate observations from at least 3 separate, comparable experiments (i.e. $n \geq 3$), and expressed as s.e.mean.

Semilogarithmic concentration-effect curves were constructed as least square non-linear fits, utilising the computer software GraphPad Prism[®] (version 3.03 for Windows[®], GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). The Hill slope factor was set at 1, and the bottom constant as 100%. Where data of concentration-effect curves are expressed as percent of control without drug, no statistical significant differences were found in the control values of second messenger accumulation among the different pre-treatments with each irreversible antagonist.

Student's two-tailed, unpaired t test was implemented to compare the $E_{\max}$, pEC_{50} and pK_i values. All reported P values are after the Bonferroni correction for multiple

comparisons (when appropriate), where a value of $P < 0.05$ was taken as statistically significant.

3.2.7 Chemicals

[2-³H]-adenine (19-23 Ci mmol⁻¹), [*O*-methyl-³H]-yohimbine (83-92 Ci mmol⁻¹) and *myo*-[2-³H]-inositol (17 Ci mmol⁻¹) were purchased from Amersham Pharmacia Biotech (U.K.). [*N*-methyl-³H]-4-DAMP (80.5 Ci mmol⁻¹) were purchased from NEN Life Science Products (Boston, MA, U.S.A.).

4-DAMP mustard hydrochloride, atropine sulphate, ATP, benextramine tetrahydrochloride, cAMP, forskolin, HEPES, IBMX, LiCl, acetyl- β -methylcholine chloride (methacholine chloride), phenoxybenzamine hydrochloride, TCA, UK 14,304 and yohimbine hydrochloride were purchased from Sigma Chemical (St Louis, MO, U.S.A.). Formic acid was purchased from Saarchem-Holpro Analytic (Krugersdorp, Gauteng, South Africa).

3.3 Results

3.3.1 Specific binding before and after pre-treatment with the irreversible antagonists, with or without receptor protection

Figure 3-1A and *Figure 3-1B* depict the specific binding of [*O*-methyl-³H]-yohimbine to α_{2A} -adrenoceptors in α_{2A} -H cells before and after pre-treatment with different concentrations of phenoxybenzamine for 20 minutes, either without protection of the α_{2A} -adrenoceptors (0 M yohimbine) or with protection of the α_{2A} -adrenoceptors (10 μ M yohimbine). It can be seen in *Figure 3-1A* that increasing concentrations of phenoxybenzamine progressively decreased specific binding (i.e. decreased α_{2A} -adrenoceptor concentration) ($P < 0.001$ for comparison of all bars). This decrease in specific binding is not seen in *Figure 3-1B* ($P > 0.05$).

Likewise, *Figure 3-1C* and *Figure 3-1D* depict the specific binding of [*O*-methyl-³H]-yohimbine to α_{2A} -adrenoceptors in α_{2A} -H cells before and after pre-treatment with

different concentrations of benextramine for 20 minutes, either without protection of the α_{2A} -adrenoceptors (0 M yohimbine), or with protection of the α_{2A} -adrenoceptors (10 μ M yohimbine). It can be seen in *Figure 3–1C* that increasing concentrations of benextramine progressively decreased specific binding ($P \leq 0.01$ for comparison of all bars, except for comparison of bars c_3 and c_4 where $P = 0.07$). This prominent decrease in specific binding is not seen in *Figure 3–1D* ($P > 0.05$ for comparison of bars, except for comparison of bars d_1 and d_3 or of bars d_1 and d_4 where $P = 0.02$ or $P = 0.01$, respectively). From the bars in *Figure 3–1B* and *Figure 3–1D* (when compared to the corresponding bars in *Figure 3–1A* and *Figure 3–1C*) it follows that 10 μ M yohimbine prevented the irreversible antagonist from eliminating the α_{2A} -adrenoceptors.

Similarly, *Figure 3–1E* and *Figure 3–1F* depict the specific binding of [*N*-methyl- ^{3} H]-4-DAMP to mACh receptors in SH-SY5Y cells before and after pre-treatment with different concentrations of 4-DAMP mustard for 20 minutes, either without protection of the mACh receptors (0 M atropine), or with protection of the mACh receptors (10 μ M atropine). It can be seen in *Figure 3–1E* that 4-DAMP mustard decreased specific binding ($P < 0.01$ for comparison of bars e_1 and e_2 or of bars e_1 and e_3). The difference in specific binding between 10 nM and 100 nM 4-DAMP mustard was not statistically significant ($P = 0.11$ for comparison of bars e_2 and e_3). From the bars in *Figure 3–1F* (when compared to the corresponding bars in *Figure 3–1E*) it can be seen that increasing concentrations of 4-DAMP mustard with receptor protection by atropine did not cause a similar prominent decrease in receptor number ($P > 0.05$ for comparison of bars f_1 and f_2 , of bars f_1 and f_3 , and bars f_2 and f_3) and it follows that 10 μ M atropine prevented the irreversible antagonist from eliminating the mACh receptors.

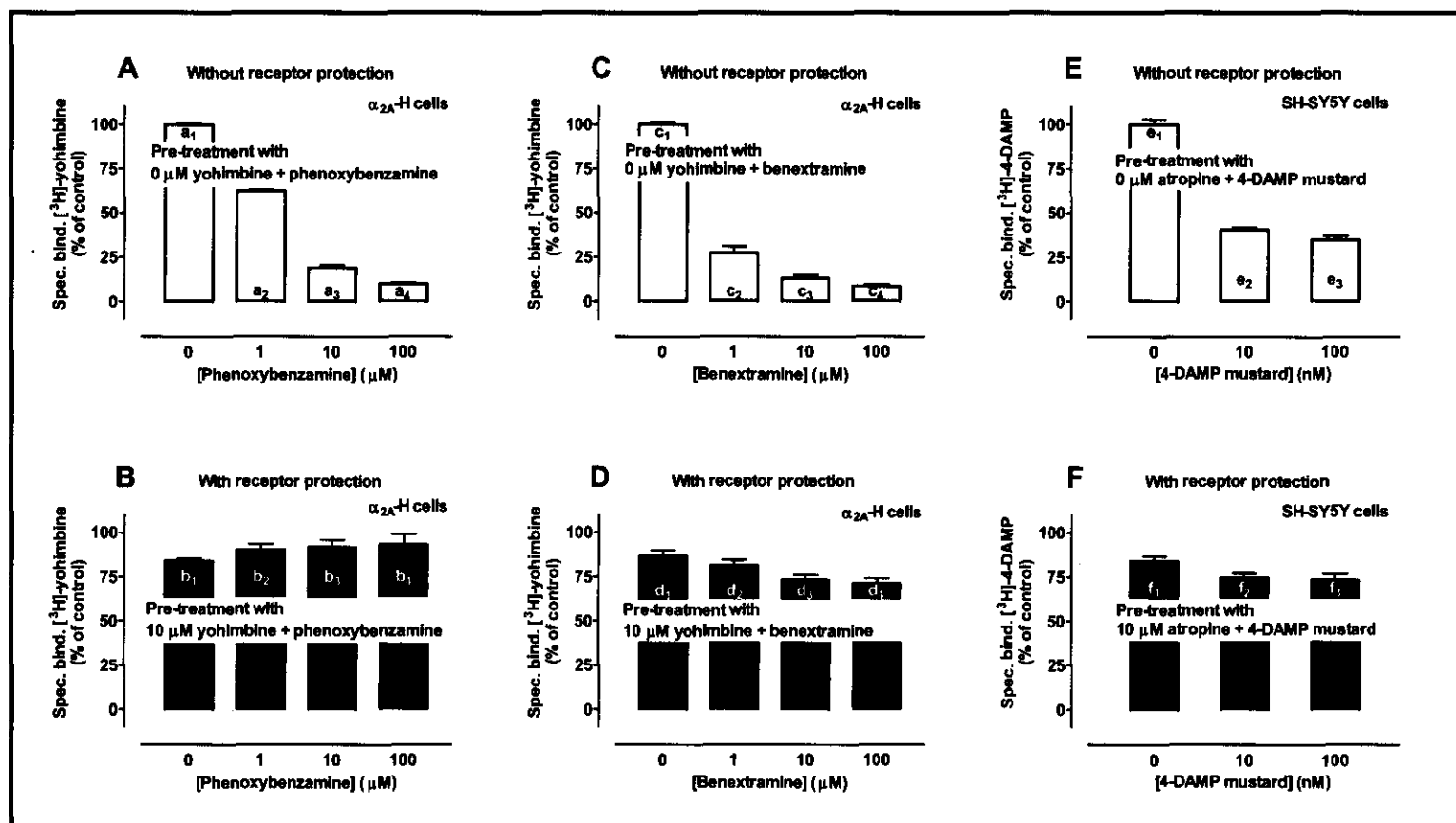


Figure 3–1: Radioligand binding studies with (A – D) 5 nM [*O*-methyl-³H]-yohimbine in α_{2A} -H cells or (E & F) 5 nM [*N*-methyl-³H]-4-DAMP in SH-SY5Y cells. Binding of the radioligand was measured after pre-treatment with phenoxybenzamine (0, 1, 10 or 100 μM ; 20 minutes) (A) without yohimbine or (B) with 10 μM yohimbine. Similarly, binding of the radioligand was measured after pre-treatment with benextramine (0, 1, 10 or 100 μM ; 20 minutes) (C) without yohimbine or (D) with 10 μM yohimbine. Radioligand binding was also measured after pre-treatment with 4-DAMP mustard (0, 10 or 100 nM; 20 minutes) (E) without atropine or (F) with 10 μM atropine. The data are averages $\pm$ s.e.mean of triplicate measurements from at least three experiments and are expressed as percent of control without drug.

3.3.2 Specific binding and second messenger formation before and after pre-treatment with the reversible antagonists

Figure 3–2A depicts the specific binding of [*O*-methyl-³H]-yohimbine to α_{2A} -adrenoceptors in α_{2A} -H cells after pre-treatment with 0 or 10 μ M yohimbine, but no irreversible competitive antagonist. Although there was a difference in specific binding of [*O*-methyl-³H]-yohimbine of about 13.4% ($P = 0.01$) between the 0 or 10 μ M yohimbine pre-treatment groups, this difference was functionally insignificant as shown by the corresponding functional data in *Figure 3–2B*, depicting the concentration-effect curves of the α_{2A} -adrenoceptor full agonist UK 14,304 in α_{2A} -H cells after the pre-treatments with 0 or 10 μ M yohimbine. These concentration-effect curves were practically superimposed with no statistically significant difference between the pEC_{50} values ($P = 0.78$) or E_{max} values ($P = 0.37$).

Likewise, *Figure 3–2C* depicts the specific binding of [*N*-methyl-³H]-4-DAMP to mACh receptors in SH-SY5Y cells after pre-treatment with 0 or 10 μ M atropine, but no irreversible competitive antagonist. Although there was a difference in specific binding of [*N*-methyl-³H]-4-DAMP of about 16% ($P = 0.02$) between the 0 or 10 μ M atropine pre-treatment groups, this difference was functionally insignificant as shown by the corresponding functional data in *Figure 3–2D*, depicting the concentration-effect curves of the mACh receptor full agonist methacholine in SH-SY5Y cells after pre-treatments with 0 or 10 μ M atropine. The concentration-effect curves were practically superimposed with no statistically significant difference between the pEC_{50} values ($P = 0.50$) or E_{max} values ($P = 0.76$).

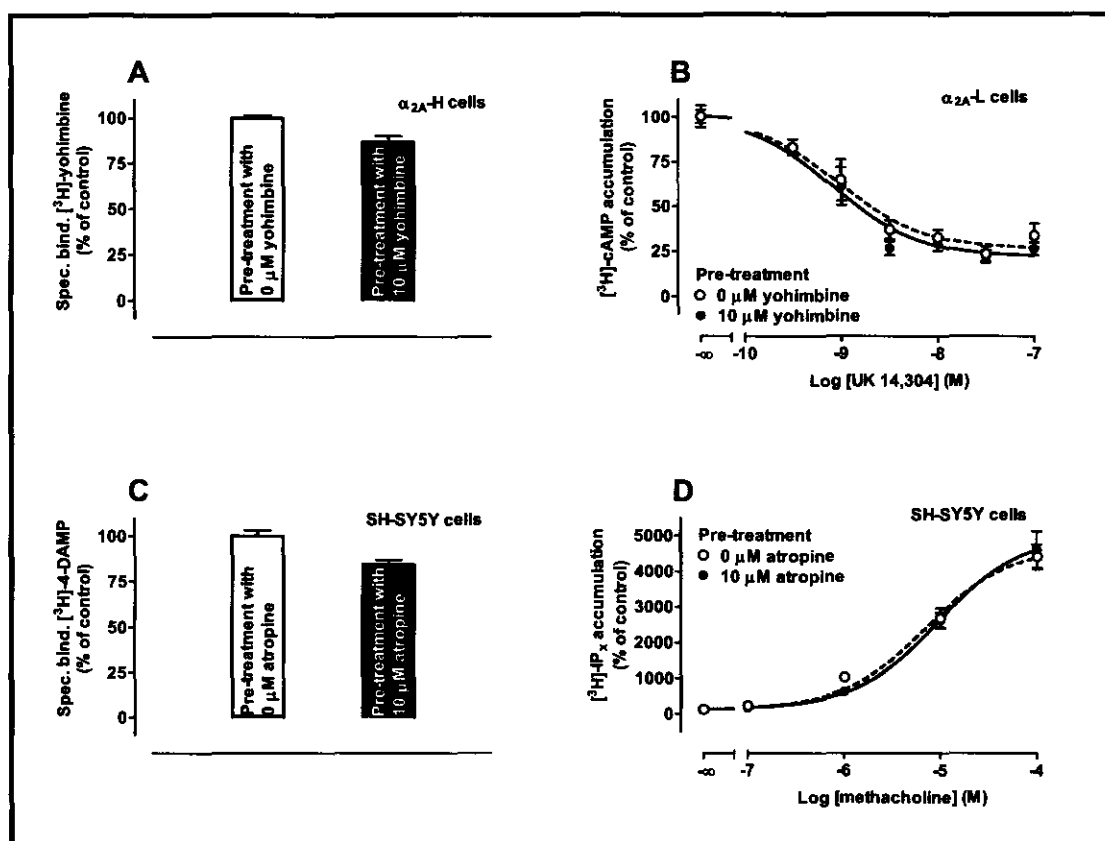


Figure 3–2: Radioligand binding and functional studies after pre-treatment with the appropriate reversible antagonist, followed by the described rinsing and incubation procedures. **(A)** Specific binding of 5 nM [*O*-methyl- ^{3} H]-yohimbine in α_{2A} -H cells after pre-treatment with yohimbine (0 or 10 μ M). **(B)** Semilogarithmic concentration-effect curves of UK 14,304 in α_{2A} -L cells by measuring whole-cell [^{3} H]-cAMP accumulation after pre-treatment with yohimbine (0 or 10 μ M). **(C)** Specific binding of 5 nM [*N*-methyl- ^{3} H]-4-DAMP in SH-SY5Y cells after pre-treatment with atropine (0 or 10 μ M). **(D)** Semilogarithmic concentration-effect curves of methacholine in SH-SY5Y cells by measuring whole-cell [^{3} H]-IP_x accumulation after pre-treatment with atropine (0 or 10 μ M). The data are averages $\pm$ s.e.mean of triplicate measurements from at least three experiments and are expressed as percent of control without drug. The concentration-effect curves (**B & D**) are non-linear least square fits.

3.3.3 Agonist-mediated effects before and after pre-treatment with the irreversible antagonists with or without specific receptor protection

Figure 3–3A depicts concentration-effect curves of the α_{2A} -adrenoceptor full agonist UK 14,304 in α_{2A} -L cells after pre-treatment with different concentrations of phenoxybenzamine for 20 minutes. As the concentration of the phenoxybenzamine was increased, the concentration-effect curves of UK 14,304 shifted parallel to the right (1 and 10 μ M), and at the highest concentration (100 μ M) the $E_{\max}$ value was

suppressed ($E_{\max}$ a_4/a_1 ratio = 0.37, $P < 0.05$). Similarly, *Figure 3–3C* depicts concentration-effect curves of UK 14,304 in α_{2A} -L cells after pre-treatment with different concentrations of benextramine for 20 minutes. As the concentration of the benextramine was increased, the concentration-effect curves of UK 14,304 initially shifted parallel to the right (1 μ M benextramine: EC_{50} shift c_2/c_1 ratio = 23, $P < 0.05$), and at higher concentrations (10 and 100 μ M) the $E_{\max}$ value was suppressed ($E_{\max}$ c_3/c_1 ratio = 0.56, $P < 0.05$; $E_{\max}$ c_4/c_1 ratio = 0.29, $P < 0.05$). However, when the α_{2A} -adrenoceptors were protected from binding to the irreversible competitive antagonist, the antagonism by the irreversible competitive antagonist was eliminated or reduced, as illustrated in *Figure 3–3B* and *Figure 3–3D*. For phenoxybenzamine, the highest concentration (100 μ M) showed a statistically significant, although small, rightward shift of the concentration-effect curve (EC_{50} shift b_4/b_1 ratio = 3.1, $P < 0.05$). For benextramine the highest concentration (100 μ M) showed a relatively large rightward shift of the concentration-effect curve (EC_{50} shift d_4/d_1 ratio = 138, $P < 0.05$) and a 10 μ M concentration benextramine produced a small rightward shift of the concentration-effect curve (EC_{50} shift d_3/d_1 ratio = 6.3, $P < 0.05$), but at 1 μ M the rightward shift of the concentration-effect curve did not reach statistical significance (EC_{50} shift d_2/d_1 ratio = 1.9; $P > 0.05$). The EC_{50} values of all other curves differed statistically significantly (EC_{50} shift d_3/d_2 ratio = 3.4, $P < 0.05$; EC_{50} shift d_4/d_2 ratio = 74, $P < 0.05$; EC_{50} shift d_4/d_3 ratio = 22, $P < 0.05$).

Figure 3–3E depicts concentration-effect curves of the mACh receptor full agonist methacholine in SH-SY5Y cells after pre-treatment with different concentrations of 4-DAMP mustard for 20 minutes. The $E_{\max}$ value was statistically significantly suppressed at 100 nM 4-DAMP mustard for 20 minutes ($E_{\max}$ e_3/e_1 ratio = 0.75, $P < 0.05$), but not at 10 nM ($E_{\max}$ e_2/e_1 ratio = 0.89, $P > 0.05$). When the mACh receptors were protected from binding to the irreversible competitive antagonist, as presented in *Figure 3–3F*, the concentration-effect curves were practically superimposed.

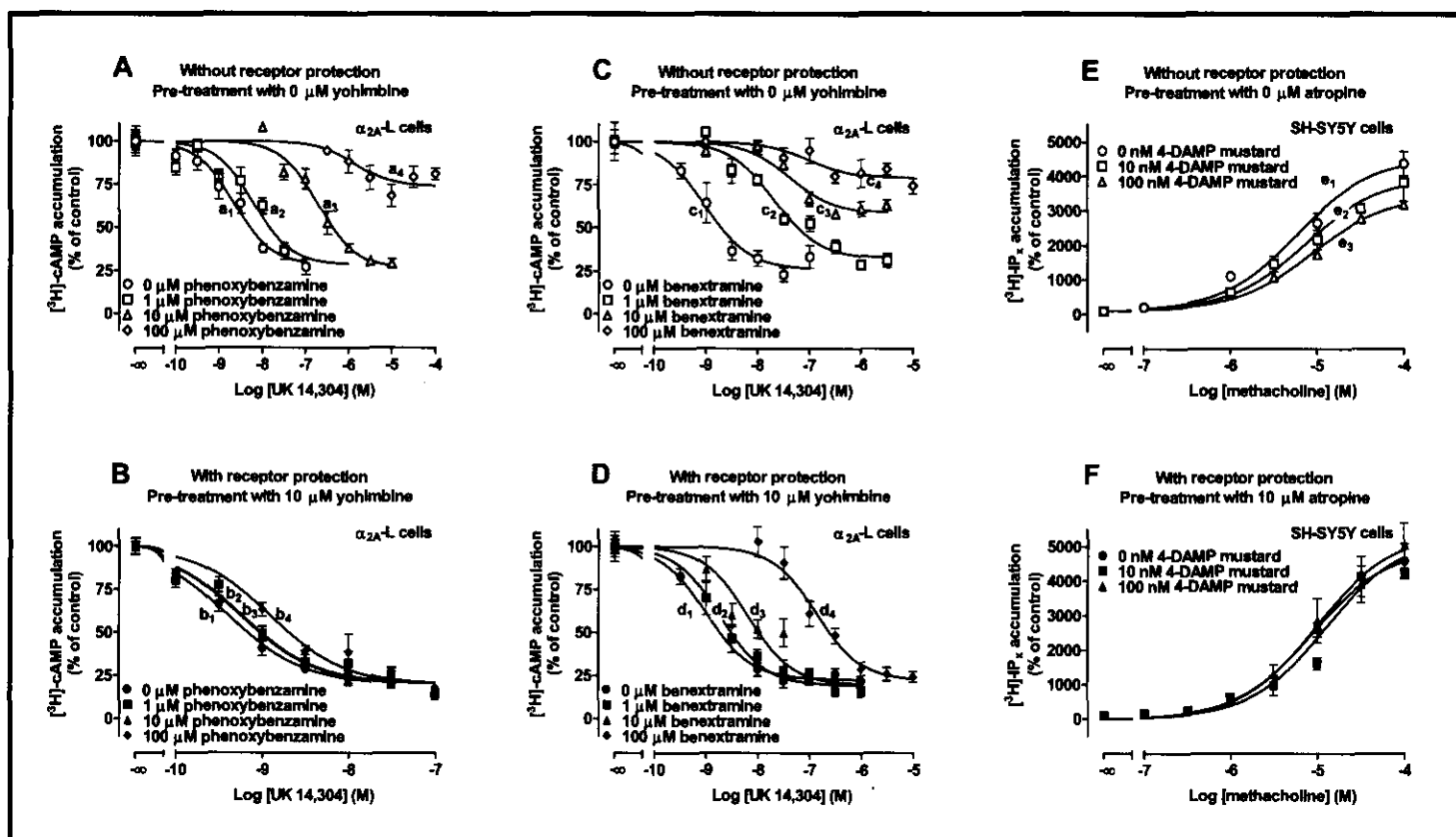


Figure 3–3: Semilogarithmic concentration-effect curves of (A – D) UK 14,304 in $\alpha_{2A}\text{-L}$ cells or (E & F) methacholine in SH-SY5Y cells. Whole-cell $[^3\text{H}]\text{-cAMP}$ accumulation measurements were performed after pre-treatment of $\alpha_{2A}\text{-L}$ cells with phenoxybenzamine (0, 1, 10 or 100 μM ; 20 minutes) (A) without yohimbine or (B) with 10 μM yohimbine. Similarly, whole-cell $[^3\text{H}]\text{-cAMP}$ accumulation measurements were performed after pre-treatment of $\alpha_{2A}\text{-L}$ cells with benextramine (0, 1, 10 or 100 μM ; 20 minutes) (C) without yohimbine or (D) with 10 μM yohimbine. Whole-cell $[^3\text{H}]\text{-cAMP}$ accumulation measurements were also performed after pre-treatment of SH-SY5Y cells with 4-DAMP mustard (0, 10 or 100 nM; 20 minutes) (E) without atropine or (F) with 10 μM atropine. The data are averages $\pm$ s.e.mean of triplicate measurements from at least three experiments and are expressed as percent of control without drug. Concentration-effect curves are non-linear least square fits.

3.3.4 Agonist-mediated effects before and after pre-treatment with benextramine for different exposure times

Figure 3–4 depicts concentration-effect curves of UK 14,304 after α_{2A} -L cells were pre-treated with identical concentrations of benextramine (10 μ M), but with increasing exposure times (20 minutes versus 60 or 120 minutes). When the α_{2A} -adrenoceptors were not protected by yohimbine (see Figure 3–4A), increasing exposure times caused a progressive rightward shift of the concentration-effect curves and concomitantly a progressive suppression of the $E_{\max}$ value. With an exposure time of 120 minutes the $E_{\max}$ was indistinguishable from zero effect. On the other hand, when the α_{2A} -adrenoceptors were protected with yohimbine (10 μ M), as depicted in Figure 3–4B, the concentration-effect curves of UK 14,304 shifted parallel to the right with a significant shift occurring at 60 minutes incubation time (EC_{50} shift b_3/b_1 ratio = 102; $P < 0.05$) and at 120 minutes incubation time (EC_{50} shift b_4/b_1 ratio = 141; $P < 0.05$). Only the EC_{50} values obtained after 60 and 120 minutes incubation, respectively, did not differ statistically significantly (EC_{50} shift b_4/b_3 ratio = 1.4, $P > 0.05$). These data therefore suggest that maximal non-specific binding already occurred when the cells were incubated with benextramine for 60 minutes.

In a single, separate experiment with triplicate observations, SH-SY5Y cells were incubated with 100 nM 4-DAMP mustard plus 10 μ M atropine for 120 minutes, instead of 20 minutes as depicted in Figure 3–3F. The $E_{\max}$ of the concentration-effect curve of the mACh receptor agonist methacholine after 120 minutes incubation time, was suppressed to 35% of the $E_{\max}$ seen at 20 minutes incubation time (data not shown). These data suggest non-specific antagonism by 4-DAMP mustard after 120 minutes incubation time.

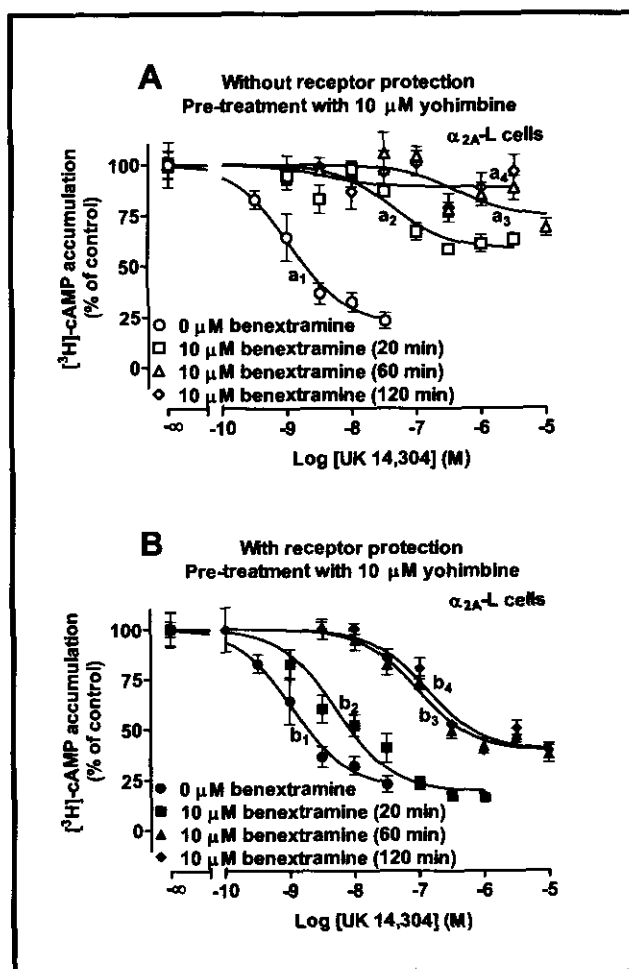


Figure 3-4: Semilogarithmic concentration-effect curves of UK 14,304 in α_{2A} -L cells. Whole-cell [^{3} H]-cAMP accumulation measurements were performed after pre-treatment of α_{2A} -L cells with benextramine (10 μ M; 20, 60 or 120 minutes) **(A)** without yohimbine or **(B)** with 10 μ M yohimbine. The data are averages $\pm$ s.e.mean of triplicate measurements from at least three experiments and are expressed as percent of control without drug. Curves are non-linear least square fits.

3.3.5 Affinity of UK 14,304 for α_{2A} -adrenoceptors before and after pre-treatment with phenoxybenzamine or benextramine

The pK_i value of the α_{2A} -adrenoceptor full agonist UK 14,304 was determined from competition binding curves against 5 nM [*O*-methyl ^{3} H]-yohimbine ($K_D = 3.67 \pm 0.00$ nM) in α_{2A} -H cells before and after pre-treatment with 100 μ M phenoxybenzamine or benextramine for 20 minutes, with α_{2A} -adrenoceptor protection by 10 μ M yohimbine. Irreversible antagonist pre-treatment did not change the α_{2A} -adrenoceptor receptor affinity for UK 14,304 and the pK_i values were determined as $pK_i = 6.64 \pm 0.05$ versus

$pK_i = 6.77 \pm 0.06$ for 0 μM or 100 μM phenoxybenzamine, respectively ($P = 0.16$), and $pK_i = 6.91 \pm 0.20$ versus $pK_i = 6.82 \pm 0.13$ for 0 μM or 100 μM benextramine, respectively ($P = 0.73$).

3.4 Discussion

The present study was undertaken to investigate whether phenoxybenzamine, benextramine and 4-DAMP mustard display any irreversible non-specific (non-competitive) mechanisms of antagonism, in addition to their known irreversible competitive antagonism.

3.4.1 The experimental conditions and pre-treatments are suitable for the evaluation of non-specific mechanisms by the irreversible competitive antagonists

As expected, it was found in the current study that phenoxybenzamine (see *Figure 3-1A*) and benextramine (see *Figure 3-1C*) decreased the α_{2A} -adrenoceptor number in α_{2A} -H cells and 4-DAMP mustard (see *Figure 3-1E*) decreased the mACh receptor number in SH-SY5Y cells when the receptors were not protected by an appropriate irreversible antagonist. When the α_{2A} -adrenoceptors in α_{2A} -H cells were protected by 10 μM yohimbine from binding to phenoxybenzamine or benextramine, the concentration of α_{2A} -adrenoceptors was not significantly reduced (see *Figure 3-1B* and *Figure 3-1D*). Similarly, when the mACh receptors in SH-SY5Y cells were protected by 10 μM atropine from binding to 4-DAMP mustard, the concentration of mACh receptors was not significantly reduced (see *Figure 3-1F*). It can therefore be concluded that, firstly, the irreversible antagonists were used at sufficient concentrations and incubation times to eliminate a significant fraction of the operational receptors in absence of receptor protection and that, secondly, the concentrations of the reversible antagonists employed were sufficient to protect the receptors from binding to the irreversible antagonists.

The washing procedure after the pre-treatments were sufficient to remove all reversibly bound yohimbine or atropine. Results in *Figure 3-2* suggest that, although

yohimbine pre-treatment caused a small but statistically significant reduction in the specific binding of [*O*-methyl-³H]-yohimbine to α_{2A} -adrenoceptors in α_{2A} -H cells, this was functionally insignificant, since concentration-effect curves of UK 14,304 at α_{2A} -adrenoceptors in α_{2A} -L cells were not influenced by the yohimbine pre-treatment (see *Figure 3–2B*). Likewise, atropine pre-treatment caused a small reduction in the specific binding of [*N*-methyl-³H]-4-DAMP to mACh receptors in SH-SY5Y cells, but the concentration-effect curves of methacholine in these cells were not influenced by atropine pre-treatment (see *Figure 3–2D*).

Therefore, the pre-treatment procedures were suitable for further identification of any irreversible non-specific mechanisms of antagonism by the respective irreversible competitive antagonists.

Importantly, Brink *et al.* (2000) showed that in control Neo cells (control cell lines for α_{2A} -H and α_{2A} -L cells, containing the selection plasmid but no α_{2A} -adrenoceptor vector) UK 14,304 elicited no effect when measuring [³H]-cAMP accumulation, suggesting that all observed UK 14,304-mediated effects in α_{2A} -H and α_{2A} -L cells are mediated *via* α_{2A} -adrenoceptors.

3.4.2 Benextramine and phenoxybenzamine, but not 4-DAMP mustard, display irreversible non-specific antagonism after 20 minutes incubation time

When receptor number in a system with spare receptors is progressively reduced by an irreversible antagonist, theory predicts that the concentration-effect curve of the agonist should progressively shift to the right until all spare receptors are eliminated, whereafter the maximal effect is reduced (Furchgott, 1966; Kenakin, 1997). The results in the current study followed this pattern, where phenoxybenzamine and benextramine caused a progressive rightward shift and suppression of the E_{max} value of the concentration-effect curves of the appropriate agonists (see *Figure 3–3A* and *Figure 3–3C*). In the absence of spare receptors, 4-DAMP mustard only suppressed the maximal effect to methacholine (see *Figure 3–3E*). Van der Graaf *et al.* (1996) proposed that if an irreversible competitive antagonist interacts only with those binding sites on the receptor macromolecule that also interact with an agonist or competitive antagonist

(syntopic binding sites), protection of these binding sites with a relative high concentration of a reversible competitive antagonist would prevent all irreversible antagonism. Therefore, if the syntopic binding sites are sufficiently protected by a reversible antagonist, the concentration-effect curves of the agonist before and after pre-treatment with a purely competitive irreversible antagonist (and appropriate washing procedure afterwards) is expected to be superimposed. We propose that any deviation from this pattern of the concentration-effect curves may be ascribed to non-specific mechanisms of the irreversible antagonist, since the irreversible antagonist cannot bind to the syntopic binding sites and any remaining antagonism must result from irreversible binding to function-modulating non-syntopic binding sites. In the present study, the concentration-effect curves of UK 14,304 in α_{2A} -L cells after pre-treatment with increasing concentrations phenoxybenzamine (see *Figure 3-3B*) or benextramine (see *Figure 3-3D*) in the presence of receptor protection by 10 μ M yohimbine, are clearly not superimposed. These data suggest that phenoxybenzamine displays a small component of irreversible non-specific antagonism at 100 μ M for 20 minutes in the α_{2A} -L system. Likewise, the data suggest that benextramine displays a small component of irreversible non-specific antagonism at 10 μ M for 20 minutes and a significant component at 100 μ M for 20 minutes in the α_{2A} -L system. According to these findings the irreversible non-specific antagonism by phenoxybenzamine is of a lesser degree than that observed with benextramine.

In contrast to phenoxybenzamine and benextramine, 4-DAMP mustard does not display irreversible non-specific antagonism at mACh receptors in SH-SY5Y cells at a concentration of up to 100 nM for 20 minutes after protection with atropine (see *Figure 3-3F*). It should, however, be emphasised that, although 4-DAMP mustard does not display irreversible non-specific antagonism at the experimental conditions employed (100 nM for 20 minutes), this observation does not rule out the possibility that it might display irreversible non-specific antagonism under different experimental conditions, i.e. when it is employed at a higher concentration and/or for a longer incubation time.

3.4.3 The irreversible non-specific antagonism by benextramine and 4-DAMP mustard is time-dependent

One of the important characteristics of irreversible competitive antagonism is the time-dependency of receptor elimination (Furchgott, 1966). Different experimental conditions (e.g. antagonist concentration, incubation time and/or biological system) may reveal non-specific mechanisms by an irreversible antagonist that did not display significant non-specific antagonism in the current study. When the incubation time of 10 μ M benextramine in α_{2A} -L was prolonged to 60 minutes or to 120 minutes (see *Figure 3–4*), the non-specific component of antagonism by benextramine increased dramatically. As a matter of fact, maximal non-specific antagonism is reached at 60 minutes incubation time (i.e. only three times the original incubation time of 20 minutes, where relatively little non-specific antagonism was observed). The same observation was made with 4-DAMP mustard in the single experiment, that did not display any non-specific antagonism at 100 nM for 20 minutes incubation time, but revealed prominent non-specific antagonism at the same concentration, but 120 minutes incubation time.

3.4.4 The non-specific antagonism by phenoxybenzamine and benextramine is not metaffinoid in nature

Both phenoxybenzamine and benextramine pre-treatments (with receptor protection by 10 μ M yohimbine) did not cause any significant change in the pK_i values of UK 14,304 at α_{2A} -adrenoceptors in α_{2A} -H cells. The data therefore suggest that phenoxybenzamine and benextramine did not change the affinity of α_{2A} -adrenoceptors for UK 14,304 and that the non-specific mechanism of antagonism of these irreversible antagonists is not metaffinoid (allosteric) in nature, i.e. receptor affinity for the agonist was not changed. The only logical alternative is that the signal transduction system of the α_{2A} -adrenoceptors was inhibited irreversibly (i.e. irreversible metactoid antagonism). It is not clear whether the results of the current study is related to the non-specific antagonism at prostanoid TP-receptors in the rat small mesenteric artery,

as reported by Van der Graaf *et al.* (1996), since their data did not distinguish the underlying mechanism of non-specific antagonism at these receptors.

3.4.5 Final conclusions and implications of the study

In conclusion, we provide here the best evidence of irreversible non-specific (metactoid) antagonism by phenoxybenzamine and benextramine at α_{2A} -adrenoceptors and by 4-DAMP mustard at mACh receptors. This non-specific antagonism is additional to their known irreversible specific (competitive) antagonism. Importantly, we have been able to reveal the non-specific mechanism of action by implementing receptor protection studies and a combination of both receptor-radioligand binding and functional assays. It is obvious from these results that mere confirmation of specific binding by the irreversible antagonist (by e.g. receptor-radioligand binding studies) does not rule out non-specific mechanisms. It should be noted that the use of relatively low concentrations of an irreversible antagonist does not exclude the possibility of additional non-specific effects. Incubation time also plays a crucial role in the extent of non-specific antagonism when applicable. The implications of this finding are highly relevant. We suggest that irreversible antagonists (and in particular benextramine and phenoxybenzamine) must be utilised with great care for the implementation of the Furchgott analysis (or any other procedure where only specific receptor elimination is intended. In particular, irreversible non-specific effects may influence the interpretation of the results without prior verification of pure competitive antagonism under the experimental conditions used. Finally, we propose that, in addition to the inactivation of adrenergic receptors by phenoxybenzamine, irreversible non-specific (metactoid) antagonism may potentially contribute to its therapeutic effect in the treatment of phaeochromocytoma. The specificity, scope or clinical significance of this mechanism may need further investigation.

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***Benextramine is an
Irreversible Non-specific
Inhibitor of Several G Protein-
coupled Receptors that Signal
through G_i, G_s and G_q***

Chapter 4

Full Paper

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Non-standard abbreviations

[^3H]-4-DAMP, [*N*-methyl- ^3H]-4-diphenylacetoxy-*N*-methylpiperidine; [^{35}S]-GTP γ S, [^{35}S]-guanosine 5'-3-*O*-(thio)triphosphate; $\alpha_{2A}\text{-H}$, α_{2A} -adrenoceptors expressed at relative high numbers; ATP, adenosine triphosphate; BSA, bovine serum albumin; cAMP, adenosine 3',5'-cyclic monophosphate; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMEM, minimum essential medium (Earle's base); FBS, fetal bovine serum; GTP γ S, 5'-(3-*O*-thio)triphosphate; GPCR, G protein-coupled receptor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid); IBMX, 3-isobutyl-1-methylxanthine; IP $_x$, total inositol phosphates; PBS, phosphate buffered saline; PTX, pertussis toxin; SH-SY5Y, cultured human neuroblastoma cells; 5HT $_{2A}$ -SH-SY5Y, cultured human neuroblastoma cells transfected with the human 5HT $_{2A}$ receptor; TCA, trichloroacetic acid; TME buffer, Tris-MgCl $_2$ -EDTA buffer (50 mM Tris, 10 mM MgCl $_2$, 1 mM EDTA); TMN buffer, Tris-MgCl $_2$ -NaCl buffer (20 mM Tris, 25 mM MgCl $_2$, 100 mM NaCl); Tris, 2-amino-2-hydroxymethyl-propan-1,3-diol; UK 14,304, 5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine (brimonidine).

Abstract

Recent data from our laboratory (see *Chapter 3*) suggested that benextramine displays irreversible non-specific antagonism at α_{2A} -adrenoceptors in addition to its irreversible binding to these receptors. In this study, we extended our investigation into the mechanism of the observed non-specific antagonism by benextramine, also including its effects at other G protein-coupled receptors (GPCRs) and G proteins.

We employed Chinese hamster ovary (CHO-K1) cells transfected to express the porcine α_{2A} -adrenoceptor at high levels (α_{2A} -H), human neuroblastoma (SH-SY5Y) cells endogenously expressing muscarinic acetylcholine (mACh) receptors, and SH-SY5Y cells transfected to express human 5HT_{2A}-serotonin receptors (5HT_{2A}-SH-SY5Y). Cells were pre-treated with appropriate concentrations of benextramine with or without an appropriate reversible competitive antagonist at a sufficient concentration to protect the receptors. This was followed by sufficient washing steps, whereafter receptor function was evaluated by measuring whole-cell [³H]-cAMP or [³H]-IP_x accumulation, or the binding of [³⁵S]-GTP γ S to prepared cell membrane proteins. In addition, we investigated the effects of benextramine on the constitutive [³⁵S]-GTP γ S binding to the purified biologically active subunit of G_o.

The results of this report suggest that benextramine acts as a non-specific irreversible antagonist at α_{2A} -adrenoceptors by inhibiting the signaling at the receptor G_i coupling, as well as by inhibiting G_s-mediated signaling. In addition, benextramine acts as a non-specific irreversible antagonist at mACh- and 5HT_{2A}-receptors signaling through G_q, presumably by binding to an allosteric binding site at these receptors and thereby inhibiting the binding of ligands. Benextramine may prove to be a useful experimental tool in investigating the signaling mechanisms of GPCRs.

4.1 Introduction

The tetramine disulfide, benextramine, has been shown to irreversibly inhibit α_2 -adrenoceptors (Taouis et al., 1987), 5HT_{1A}-serotonergic receptors (Stanton and Beer, 1997), H₂-histaminergic receptors (Belleau et al., 1982), and neuropeptide Y-receptors (Melchiorre et al., 1994). Present knowledge regarding the mechanism of action of benextramine is limited to the observations from relatively few studies, predominantly radioligand binding studies. It is generally assumed that benextramine irreversibly inactivates the receptors by reducing the number of receptors available for binding to its ligands, presumably by binding to the ligand binding site. Classical irreversible antagonists such as the haloalkylamines (e.g. phenoxybenzamine) are believed to form covalent bonds between a highly reactive intermediate ethyleneimmonium (aziridinium) ion and electron donor groups on the receptor. Benextramine, however, works differently and it has been proposed that benextramine inactivates receptors *via* a disulfide-thiol interchange reaction between the molecule and distinct sites on the receptor (Brasili et al., 1986; Giardinà et al., 1996; Melchiorre, 1981). Besides having many additional experimental applications in pharmacology, benextramine has been employed, for example, to investigate receptor turnover after irreversible inhibition (Taouis et al., 1987), receptor sequestration in compartments and their movement to cell surfaces (McKernan et al., 1988), and in Furchgott's analysis to determine equilibrium dissociation rate constants for agonists after decreasing receptor number to eliminate spare receptors, presumably without influencing subsequent signal transduction (Brink et al., 2000; Kenakin, 1997; Stanton and Beer, 1997; Umland et al. 2001). There is one report, however, suggesting that benextramine acts as an irreversible, non-specific (non-competitive) antagonist at prostanoid TP-receptors. The nature of this mechanism was not investigated, but it has been suggested that benextramine interferes irreversibly with signal transduction of the receptor or reduces receptor affinity for the agonist (Van der Graaf et al., 1996).

In addition, the results of a recent study in our laboratory (see *Chapter 3*) suggest that, besides for decreasing the specific number of α_{2A} -adrenoceptors (as measured by radiolabeled ligand studies), benextramine at 10 or 100 μ M for 20, 60 or 120 minutes also displays irreversible non-specific antagonism at these receptors. The latter was concluded from functional studies, measuring α_{2A} -adrenoceptor agonist-induced

inhibition of cAMP accumulation, after these receptors were protected from binding to benextramine with a high concentration of a reversible antagonist. In the same study we also showed that the affinity of the agonist in whole cells was not modified by the non-specific antagonistic property of benextramine, so that it was concluded that benextramine irreversibly influences signal transduction (i.e. non-specific signal transduction antagonism). We also postulated that the possible non-specific binding sites of benextramine could include the effector region of the α_{2A} -adrenoceptor (affecting receptor G_i protein coupling efficiency), the G_i protein complex itself (affecting G_i protein function), the G_i protein effector site (affecting G_i protein adenylyl cyclase coupling efficiency), or adenylyl cyclase itself (affecting enzyme activity). This hypothesis formed the basis of the current study, where we tried to resolve the non-specific antagonism by benextramine. In addition, we investigated whether signal transduction pathways *via* other G protein types may also be inhibited by benextramine.

In the current study we have been able to show that benextramine displays its non-specific antagonism by binding at a site affecting the α_{2A} -adrenoceptor G_i protein coupling or G protein function, without affecting the GTP-binding capacity of the G protein. In addition, we have been able to show that benextramine also irreversibly inhibits in a non-specific fashion the signaling of α_{2A} -adrenoceptors through G_s proteins, as well as the signaling of muscarinic acetylcholine (mACh) and 5HT_{2A}-receptors through G_q proteins.

4.2 Materials and methods

4.2.1 Radiochemicals

[2-³H]-adenine (19-23 Ci/mmol), [³⁵S]-guanosine 5'-3-O-(thio)triphosphate ([³⁵S]-GTP γ S) (1146 Ci/mmol), and *myo*-[2-³H]-inositol (17 Ci/mmol) were obtained from Amersham Pharmacia Biotech (U.K.). [*N*-methyl-³H]-4-diphenylacetoxy-*N*-methylpiperidine ([³H]-4-DAMP) (80.5 Ci/mmol), [ethylene-³H]-ketanserin ([³H]-ketanserin) (63.3 Ci/mmol), and [benzene ring-³H]-spiperone ([³H]-spiperone) (24.9 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, U.S.A.).

4.2.2 Cell culture media

Dulbecco's modified Eagle's medium (DMEM):Ham's F-12 (1:1 ratio mixture) and G-418 was obtained from BioWhittaker (Rockville, ME, U.S.A.). Bovine serum albumin (BSA), DMEM, fetal bovine serum (FBS), Ham's F-12, minimum essential medium (Earle's base) (EMEM), penicillin-streptomycin mixture and trypsin-versene mixture were obtained from Highveld Biological (Lyndhurst, Gauteng, South Africa). DOTAP liposomal transfection reagent was obtained from Roche Diagnostics (Mannheim, Germany).

4.2.3 $G_o\alpha$ protein

The biologically active, purified myristoylated preparation of the rat recombinant $G_o\alpha$ -subunit (25 μ g/12.5 μ l buffered solution) was obtained from Calbiochem (San Diego, CA, U.S.A.).

4.2.4 Other chemicals

2-amino-2-hydroxymethyl-propan-1,3,-diol (Tris) was obtained from Acros (Geel, Belgium). Fractionated BSA was obtained from Boehringer Mannheim (Mannheim, Germany). Ascorbic acid, Bradford reagent, ethylenediaminetetraacetic acid (EDTA), KCl, KH_2PO_4 , $MgCl_2$, NaCl and Na_2HPO_4 were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Saarchem-Holpro Analytic (Krugersdorp, Gauteng, South Africa). 3-isobutyl-1-methylxanthine (IBMX), 5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine (brimonidine or UK 14,304), 5-hydroxytryptamine creatinine sulfate complex (serotonin creatinine sulfate), 5'-(3-*O*-thio)triphosphate (GTP γ S), acetyl- β -methylcholine chloride (methacholine chloride), adenosine 3',5'-cyclic monophosphate (cAMP), adenosine triphosphate (ATP), atropine sulfate, benextramine tetrahydrochloride (dithiobis(*N*-[*N*-(2-methoxybenzyl)-6-aminohexyl]-2-aminoethane)), dithiothreitol (DTT), forskolin, guanine diphosphate (GDP), LiCl, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (HEPES), nonaethylene glycol monododecyl ether (Brij[®] or Lubrol[®]), pertussis toxin (PTX), ritanserin, trichloroacetic acid (TCA), and yohimbine hydrochloride were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

4.2.5 Cultured cells

In the present study, we have employed three cultured cell lines. The first cell line was derived from a Chinese hamster ovary (CHO-K1) cell line, transfected to express relative high numbers of the wild-type porcine α_{2A} -adrenoceptor (cell line denoted α_{2A} -H). This cell line was a kind gift from Dr Rick Neubig (Departments of Pharmacology and Internal Medicine (Division of Hypertension), University of Michigan, Ann Arbor, MI, U.S.A.). The pharmacological profile and receptor expression characteristics of the α_{2A} -H cell line were characterized previously and the α_{2A} -adrenoceptor concentration was reported as 19 ± 2 pmol/mg membrane protein (Brink et al., 2000). The α_{2A} -H cells were employed to determine the effect of benextramine pre-treatment on α_{2A} -adrenergic receptors by determining agonist-stimulated, G_s -mediated activation of adenylyl cyclase, measuring [^{3} H]-cAMP accumulation. Membranes from these cells were used to determine the effect of benextramine pre-treatment on agonist-induced, G_i -mediated specific binding of [^{35} S]-GTP γ S. The α_{2A} -H cells were maintained and grown to 95% confluency in a humidified environment at 37°C with 5% CO $_2$ in 150 cm ^{2} cell culture flasks with Ham's F-12 medium containing 10% v/v fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml fungizone and 400 μ g/ml geneticin (G-418).

The second cell line was a human neuroblastoma (SH-SY5Y) cell line, obtained from Highveld Biological (Lyndhurst, Gauteng, South Africa), originally from the American Type Culture Collection (U.S.A.). SH-SY5Y cells endogenously express predominantly M $_3$ -muscarinic acetylcholine receptors (mACh receptors) (Slowiejko et al., 1996), with some evidence for M $_1$ - and M $_2$ -mACh receptors (Kukkonen et al., 1992). The SH-SY5Y cells were employed to determine the effect of benextramine pre-treatment on mACh receptors by determining agonist-stimulated, G_q -mediated activation of phospholipase C, measuring total [^{3} H]-inositol phosphates ([^{3} H]-IP $_x$) accumulation. These cells were also employed for radioligand binding studies to investigate the effect of benextramine on the mACh receptor concentration. SH-SY5Y cells were maintained and grown to 95% confluency as previously described for the α_{2A} -H cells, but with a 1:1 ratio mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) and without G-418.

The third cell line employed was SH-SY5Y cells transfected with the human 5-hydroxytryptamine-2A ($5HT_{2A}$) receptor, denoted $5HT_{2A}$ -SH-SY5Y cells (see transfection protocol below). The human $5HT_{2A}$ plasmid cDNA in the pIRES (Neo^r) mammalian expression vector was kindly provided by Dr Brian Roth (Department of Biochemistry, Case Western Reserve University, Cleveland, OH, U.S.A.). Human neuroblastoma SH-SY5Y cells were transfected with the vector using DOTAP liposomal transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Since G-418 resistance was included in the transfection, the cells were subjected to G-418 (400 μ g/ml) treatment after 48 hours and the surviving colony was harvested and implemented in this study. Transfected cells were not cloned, but the transfection mix used. Successful transfection was confirmed pharmacologically by concentration-effect curves with the $5HT_{2A}$ receptor agonist serotonin (with control SH-SY5Y cells giving no serotonin-induced effect, data not shown) and by the observed K_D value of [^{3} H]-spiperone at $5HT_{2A}$ -receptors ($K_D = 8.3 \pm 3.0$ nM, $B_{max} = 3,166 \pm 421$ receptors/cell) as determined from saturation binding curves, using 10 μ M ritanserin to define non-specific binding. This K_D value corresponds with reported K_i values for [^{3} H]-spiperone at $5HT_{2A}$ -receptors against various radioligands, ranging between 0.12 nM (Roth et al., 1987) and 50.11 nM (Boess and Martin, 1994). The $5HT_{2A}$ -SH-SY5Y cells were employed to determine the effect of benextramine pre-treatment on $5HT_{2A}$ receptors by determining agonist-stimulated, G_q -mediated activation of phospholipase C, by measuring [^{3} H]-IP_x accumulation. These cells were also employed for radioligand binding studies to investigate the effect of benextramine on $5HT_{2A}$ receptor concentration. The cells were maintained and grown to 95% confluency as the SH-SY5Y cells, but with 400 μ g/ml G-418.

4.2.6 Preparation and benextramine pre-treatment of cells

In preparation for experiments, the α_{2A} -H, SH-SY5Y or $5HT_{2A}$ -SH-SY5Y cell suspensions were prepared in the indicated culture medium (as described above) and 100 ng/ml pertussis toxin (PTX) was added when indicated. The cells were seeded in 24-well plates at a density of roughly 3×10^6 cells/ml for α_{2A} -H cells and 6×10^6

cells/ml for SH-SY5Y and 5HT_{2A}-SH-SY5Y cells. When cell pre-treatments were intended for membrane preparation, the pre-treatments were performed directly in the 150 cm² culture flasks. Cells attached adequately to the well bottoms, allowing several aspirations and new additions of medium without significant cell loss (confirmed by microscopic observations). When appropriate, labeling of the cells were also performed as described below for the [³H]-cAMP and [³H]-IP_x assays. Pre-treatments were initiated by rinsing the cells once with phosphate buffered saline (PBS, consisting of 0.02% w/v KCl, 0.02% w/v KH₂PO₄, 0.8% w/v NaCl and 0.09% w/v Na₂HPO₄) at 37°C. The following washing and incubation steps were followed for α_{2A} -H, SH-SY5Y or 5HT_{2A}-SH-SY5Y cells: (1) Cells were incubated with either 0 M or 10 μ M (>1,000 $\times$ K_i value) of the appropriate reversible competitive antagonist in DMEM for 30 minutes at 37°C and 5% CO₂. For α_{2A} -adrenoceptors we used yohimbine ($K_D = 3.67 \pm 0.00$ nM), for mACh receptors we used atropine (reported average $K_i = 0.50$ nM for M₃-mACh receptors – Hirose et al. (2001)) and for 5HT_{2A}-receptors we used ritanserin (reported average $K_i = 0.25$ nM – Bonhaus et al. (1997)) in DMEM for 30 minutes at 37°C and 5% CO₂. (2) Thereafter, the cells were incubated with either 0 M or 10 μ M of the appropriate competitive antagonist plus 0 or 100 μ M benextramine (freshly prepared) for 20 minutes at 37°C and 5% CO₂. (3) Cells were then rinsed twice with PBS, and incubated twice for 20 minutes with pure DMEM at 37°C and 5% CO₂ to allow dissociation of any reversibly bound drugs. Hereafter the assays for whole-cell [³H]-cAMP or [³H]-IP_x accumulation or radioligand binding were conducted or membranes were prepared (procedures described below).

4.2.7 Preparing membranes from α_{2A} -H cells

After the appropriate pre-treatment of whole α_{2A} -H cells (see above), the cells were washed twice with PBS, the cell monolayer was loosened with ethylenediaminetetraacetic acid (EDTA) in PBS (0.02% w/v), and the cells scraped from the culture flask surface with a cell scraper. The cell suspension was centrifuged in a bench top centrifuge (5,411 $\times$ g, 4°C, 15 minutes), the supernatant discarded and the pellet washed twice with ice-cold PBS, whereafter the pellet was re-suspended in 1 mM Tris buffer (pH 7.4). The cell suspension was tumbled for 15 minutes at 4°C, homogenized with a Teflon[®] homogenizer, and centrifuged at 1,000 $\times$ g in a Beckman

ultracentrifuge at 4°C for 15 minutes. The supernatant was collected and kept on ice, while the pellet was re-suspended in the Tris buffer and the preceding procedure repeated to collect all protein. The resulting supernatants were centrifuged at $40,000 \times g$ in a Beckman ultracentrifuge at 4°C for 60 minutes. The resulting pellet was re-suspended and homogenized in TME buffer (50 mM Tris, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.4). Protein concentrations were determined with the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard and determining absorbance with a 96-well plate reader and a 560 nm filter (Labsystems Multiskan RC). Snap-frozen aliquots were stored at -86°C for up to 4 weeks.

4.2.8 Measuring [^{35}S]-GTP γ S binding in α_{2A} -H cell membranes

The [^{35}S]-GTP γ S binding assay to α_{2A} -H membranes was based on the procedures described by Sternweis and Robishaw (1984) and Yang and Lanier (1999), but adapted for the present study. Immediately before the [^{35}S]-GTP γ S binding assays, the membranes were thawed on ice and the protein concentrations adjusted to 1.4 $\mu g/\mu l$ by adding the appropriate volume of TME dilution buffer. Freshly prepared assay buffer (0.5 nM [^{35}S]-GTP γ S, 1 μM guanine diphosphate (GDP), 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol (DTT), pH 7.4, at 4°C) was used to prepare a concentration range of UK 14,304. In each test tube 90 μl of the assay buffer containing the appropriate concentration of UK 14,304 was added and heated for 5 minutes at 25°C in a water bath. Thereafter 10 μl membrane was added and incubated for 40 minutes at 25°C in the water bath. After the incubation, 3 ml ice-cold TMN washing buffer (20 mM Tris, 25 mM $MgCl_2$, 100 mM NaCl, pH 7.4) was added to each membrane sample and it was immediately filtered through Whatman GF/C filters (Kent, U.K.), using a Hoefer filtration apparatus under vacuum. Each sample was washed thrice with ice-cold TMN buffer. Non-specific binding of [^{35}S]-GTP γ S was defined by samples with assay buffer but no membrane protein. Filters were air-dried, whereafter radioactivity was determined by liquid scintillation counting. The specific UK 14,304-induced binding of [^{35}S]-GTP γ S to the membranes was determined by subtracting the non-specific binding from the total binding.

4.2.9 Assessment of binding of [^{35} S]-GTP γ S to $G_o\alpha$

The [^{35} S]-GTP γ S binding assay to $G_o\alpha$ was based on the procedures described by Graber et al. (1992) and Sternweis and Robishaw (1984), but adapted to accommodate the pre-treatment with benextramine. The $G_o\alpha$ protein was diluted to a concentration of 8 ng/ μ l with a sample dilution buffer (10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1% w/v nonaethylene glycol monododecyl ether, pH 8.0) and kept on ice. Before measuring the binding of [^{35} S]-GTP γ S to $G_o\alpha$, the protein was pre-treated with either 0 M or 100 μ M benextramine for 2 hours at 4°C, or for 30 minutes at 25°C. Immediately after pre-treatment, the benextramine pre-treatment groups were divided into 10 μ l samples in test tubes and 10 μ l dilution buffer was added on ice. Thereafter 20 μ l binding cocktail (0.8 nM [^{35} S]-GTP γ S, 2 μ M GTP γ S in 50 mM HEPES, 1 mM EDTA, 40 mM MgCl₂, 200 mM NaCl, 1 mM DTT, pH 8.0) was added to each test tube at the indicated temperature (4°C or 25°C). After incubation, 3 ml ice-cold TMN wash buffer (pH 8.0) was added to each sample, and the bound [^{35} S]-GTP γ S separated from the free fraction by rapid filtration through type HAWP nitrocellulose membrane filters (Millipore, Bedford, MA, U.S.A.), placed on a Hoefer filtration apparatus under vacuum. Each sample was washed thrice with ice-cold TMN buffer. Non-specific binding of [^{35} S]-GTP γ S was defined by samples with assay buffer but no $G_o\alpha$ protein. Filters were air-dried, whereafter radioactivity was determined by liquid scintillation counting.

4.2.10 Measurement of whole-cell total [^{3} H]-cAMP accumulation

After the appropriate pre-treatment, the measurement of whole-cell G_s -mediated [^{3} H]-cAMP accumulation in α_{2A} -H cells was determined as described by Wade et al. (1999) and Wong (1994). In the present study, α_{2A} -H cells were labeled before seeding by adding 1 μ Ci [2- ^{3} H]-adenine per well for at least 18 hours before the assay was conducted. After the pre-treatment and washing procedure (as described above), the assay was initiated by adding the stimulation medium consisting of DMEM with 1 mM 3-isobutyl-1-methylxanthine (IBMX), 30 μ M forskolin, and the appropriate concentration of UK 14,304 to construct the appropriate semilogarithmic concentration-

effect curves. After a 20-minute incubation time at 37°C and 5% CO₂, the stimulation medium was aspirated and the [³H]-cAMP accumulation reaction terminated by adding 1 ml ice-cold 5% trichloroacetic acid (TCA) containing 1 mM adenosine triphosphate (ATP) and 1 mM cAMP. [³H]-cAMP accumulation is linear with time during the 20 minutes incubation period (data not shown). The plates were then left at 4°C for at least 30 minutes for the cells to lyse. The acid soluble nucleotides were separated on Dowex and alumina columns as described by Salomon et al. (1974), and the radioactivity determined by liquid scintillation counting. The [³H]-cAMP accumulation was normalized by dividing the [³H]-cAMP counts by the total [³H]-nucleotide counts. These values were then divided by the corresponding values obtained in the presence of IBMX and forskolin, but without UK 14,304 to calculate the percentage of the control.

4.2.11 Measurement of whole-cell total [³H]-IP_x accumulation

After pre-treatment with 0 or 100 μM benextramine, the measurement of whole-cell G_q-mediated [³H]-IP_x accumulation in SH-SY5Y or 5HT_{2A}-SH-SY5Y cells was done according to the principles described by Godfrey (1992), and the procedure as described by Casarosa et al. (2001) was essentially followed. In the present study, SH-SY5Y or 5HT_{2A}-SH-SY5Y cells were labeled for at least 18 hours before the assay was conducted by adding 1 μCi per well *myo*-[2-³H]-inositol in inositol-free medium (minimum essential medium, Earle's base, EMEM) with 0.5% v/v BSA. The [³H]-IP_x assay was initiated by adding the stimulation medium consisting of a mixture of DMEM, 20 mM LiCl, 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (HEPES), and the appropriate concentration of methacholine or serotonin (with 0.02% ascorbic acid) to construct the appropriate semilogarithmic concentration-effect curves. Cells were incubated with the agonist for 60 minutes at 37°C and 5% CO₂. [³H]-IP_x accumulation is linear with time beyond the 60 minutes incubation period for muscarinic receptors in SH-SY5Y cells and serotonergic receptors in 5HT_{2A}-SH-SY5Y cells (data not shown). After 60 minutes the stimulation medium was aspirated and the [³H]-IP_x accumulation reaction terminated by adding 1 ml ice-cold 10 mM formic acid to each well. The plates were then left at 4°C for at least 90 minutes for the cells to lyse. The [³H]-IP_x was separated on Dowex columns (250 μl Dowex 1 × 8-400, 200-

400 mesh, 1-chloride form per 2 ml, Bio-Rad Poly-Prep column), and the radioactivity determined by liquid scintillation counting. The [^3H]-IP_x accumulation was expressed as the percentage of the control value without the agonist.

4.2.12 Assessment of binding of radioligands to mACh- and 5HT_{2A}-receptors

SH-SY5Y or 5HT_{2A}-SH-SY5Y cells were plated, pre-treated and washed according to the procedures described above, and then rinsed once with EMEM. The assay employing SH-SY5Y cells was initiated by adding 5 nM [^3H]-4-DAMP to determine total binding, and non-specific binding was defined by adding 10 μM atropine to the radioligand. Likewise, the assay employing 5HT_{2A}-SH-SY5Y cells was initiated by adding 5 nM [^3H]-ketanserin, and non-specific binding was defined by adding 10 μM ritanserin to the radioligand. After a 30-minute incubation time at 37°C and 5% CO₂, the cells were rinsed once with ice-cold PBS and the reaction terminated by adding 1 ml ice-cold 5% TCA to each well. The plates were then left at 4°C for at least 30 minutes for the cells to lyse. The TCA from each well was directly transferred to scintillation vials and the radioactivity determined by liquid scintillation counting. The specific binding (and relative receptor concentrations) was determined by subtracting the non-specific binding from the total binding, and expressed as the percentage of specific binding in the control without drugs.

4.2.13 Data analysis

Data from all assays were obtained as triplicate measurements from at least three (i.e. $n \geq 3$) separate experiments, and expressed as mean $\pm$ S.E.M. Semilogarithmic concentration-effect curves were constructed as non-linear least square fits, by utilizing the computer software GraphPad Prism® (version 3.03 for Windows®, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). The Hill slope factor was set at 1, and the bottom constant as 100%. Student's two-tailed, unpaired t test was implemented to compare the E_{max} and pEC_{50} values. All reported statistical probability (p) values are after the Bonferroni correction for multiple comparisons (when appropriate), and a value of $p < 0.05$ was taken as statistically significant.

4.3 Results

4.3.1 [^{35} S]-GTP γ S binding to $G_i\alpha$ proteins in α_{2A} -H membranes after benextramine pre-treatment, with or without α_{2A} -adrenoceptor protection

Previous observations in our laboratory (see Chapter 3) suggested that benextramine displays non-specific signal-transductional antagonism at α_{2A} -adrenoceptors as determined by measuring [^{3} H]-cAMP accumulation. In the current study we therefore conducted [^{35} S]-GTP γ S binding studies to investigate whether the non-specific antagonism by benextramine is due to irreversible binding to a site at the receptor and/or G protein level, or whether the binding is located downstream to the G protein in the signal-transduction system. As before, α_{2A} -H cells were pre-treated with 0 or 100 μ M benextramine for 20 minutes at 37°C, plus 0 M yohimbine, or 10 μ M yohimbine to protect the α_{2A} -adrenoceptors from binding to benextramine. After sufficient washing procedures to remove all unbound and reversibly bound drugs, as developed and fully characterized in our laboratory before (see Chapter 3), membranes were prepared. Figure 4–1 depicts concentration-effect curves of the full α_{2A} -adrenoceptor agonist UK 14,304, measuring [^{35} S]-GTP γ S binding to G proteins in these membranes. In Figure 4–1A, a concentration-dependent effect of UK 14,304 was observed in membranes pre-treated with drug-free medium (i.e. control curve a₁, pre-treated with 0 μ M yohimbine plus 0 μ M benextramine). As expected, when the membranes were pre-treated with benextramine (i.e. curve a₂, pre-treated with 0 μ M yohimbine plus 100 μ M benextramine), the effect of UK 14,304 was totally abolished (from $E_{\max}$, $n = 3$, $p < 0.01$). When the membranes were pre-treated with yohimbine alone (Figure 4–1B, curve b₁, pre-treated with 10 μ M yohimbine plus 0 μ M benextramine), a concentration-effect curve was obtained similar to curve a₁, confirming previous results (see Chapter 3) that yohimbine is removed by the washing procedure after the pre-treatment. Importantly, however, when the α_{2A} -adrenoceptors were protected from binding to benextramine during the pre-treatment (Figure 4–1B, curve b₂, pre-treated with 10 μ M yohimbine plus 100 μ M benextramine), the concentration-effect curve of UK 14,304 was completely suppressed as was found without receptor protection, as represented in curve a₂ (from $E_{\max}$, $n = 3$, $p < 0.01$). Results suggest that the protection of α_{2A} -

adrenoceptors with yohimbine does not prevent benextramine from inhibiting the binding of [^{35} S]-GTP γ S to the membranes and therefore G_i mediated signaling through the receptor.

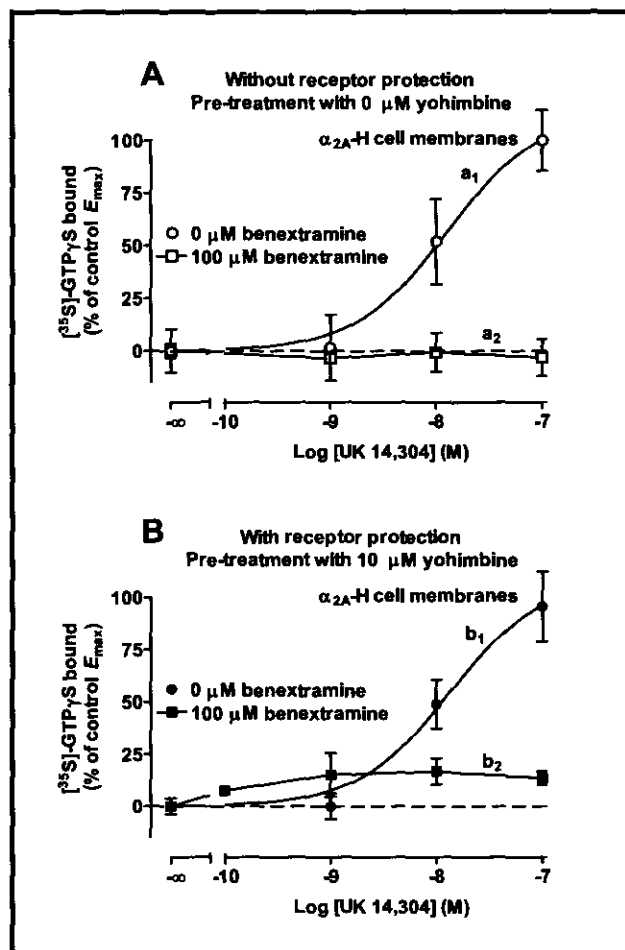


Figure 4-1: Semilogarithmic concentration-effect curves of UK 14,304 in α_{2A} -H cell membranes as measured by [^{35} S]-GTP γ S binding to endogenous G proteins in membranes. α_{2A} -H cell membranes were prepared after whole-cell pre-treatments for 20 minutes with (A) 0 or 100 μ M benextramine plus 0 M yohimbine (i.e. without receptor protection), or (B) 0 or 100 μ M benextramine plus 10 μ M yohimbine (i.e. with receptor protection). [^{35} S]-GTP γ S binding in all curves is presented as the mean $\pm$ S.E.M. and expressed as percentage of the control E_{max} of curve a₁. Data represent the average of triplicate observations of three experiments ($n = 3$). Curves a₁ and b₁ are non-linear least square fits.

4.3.2 Binding of [^{35} S]-GTP γ S to $G_o\alpha$ before and after incubation with benextramine at different incubation times and temperatures

$G_o\alpha$ is a relatively stable GTP-binding protein of the $G_{i/o}$ family and has been shown to constitutively (in the absence of receptor and agonist) bind guanine nucleotides (Sternweis and Robishaw, 1984). To investigate whether benextramine directly binds to the G protein nucleotide site to inhibit GTP binding, $G_o\alpha$ was pre-treated with 0 or 100 μ M benextramine for 120 minutes at 4°C, or for 30 minutes at 25°C, whereafter the [^{35} S]-GTP γ S binding was measured, as presented in Figure 4–2. After pre-treatment with 0 or 100 μ M benextramine for 120 minutes at 4°C, the specific binding obtained is depicted in Figure 4–2A. As can be seen from Figure 4–2A, benextramine has not significantly reduced the binding of [^{35} S]-GTP γ S to $G_o\alpha$ ($n = 3$, $p = 0.80$) and the amount of [^{35} S]-GTP γ S bound after pre-treatment with 0 or 100 μ M benextramine was measured as 17.2 ± 3.0 and 16 ± 3.1 fmol/ng membrane protein respectively. Likewise, after pre-treatment with 0 or 100 μ M benextramine for 30 minutes at 25°C, the specific binding obtained is depicted in Figure 4–2B. As can be seen from Figure 4–2B, benextramine has not significantly reduced the binding of [^{35} S]-GTP γ S to $G_o\alpha$ ($n = 3$, $p = 0.54$) and the amount of [^{35} S]-GTP γ S bound after pre-treatment with 0 or 100 μ M benextramine was measured as 2.9 ± 0.6 and 3.7 ± 1.1 fmol/ng membrane protein respectively. Although pre-treatment with benextramine at both incubation times and temperatures did not significantly decrease the binding of [^{35} S]-GTP γ S to $G_o\alpha$, overall binding is significantly lower at the shorter incubation time but higher temperature. These results suggest that incubation of $G_o\alpha$ at higher temperatures with shorter incubation times reduce the binding of [^{35} S]-GTP γ S to the nucleotide binding site of the G protein. According to Sternweis & Robishaw (1984), purified G proteins (such as $G_o\alpha$), could be stored at -80°C or even on ice (4°C) for several weeks with little or no loss of binding activity. Thus the observed overall significant reduction in binding of [^{35} S]-GTP γ S to $G_o\alpha$ after pretreatment with benextramine for 30 minutes at 25°C is due to reduced biological activity of the G protein, since it is well-known that higher temperatures cause conformational changes that may decrease or destroy its biological activity.

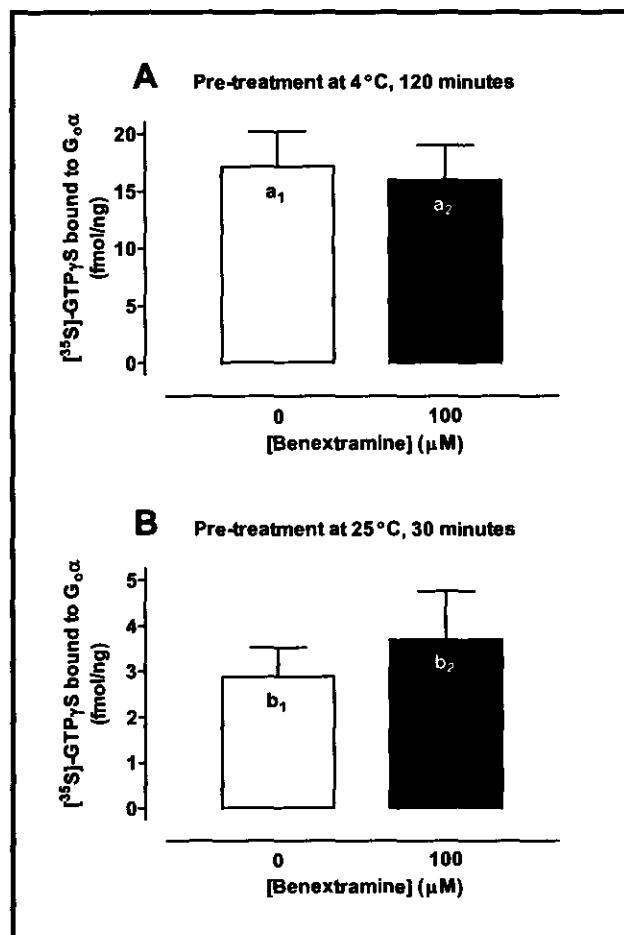


Figure 4–2: Constitutive [^{35} S]-GTP γ S binding to purified $G_{\alpha\alpha}$ protein (fmol/ng). The $G_{\alpha\alpha}$ protein was pre-treated with 0 or 100 μ M benextramine at **(A)** 4°C for 120 minutes, or **(B)** 25°C for 30 minutes before [^{35} S]-GTP γ S binding. The bar graphs represent the mean specific binding $\pm$ S.E.M and data represents the average of triplicate observations of three experiments ($n = 3$).

4.3.3 G_s -mediated [^{3} H]-cAMP accumulation in α_{2A} -H cells after pre-treatment with benextramine, with or without α_{2A} -adrenoceptor protection

Since our previous observations (see Chapter 3) investigated the non-specific antagonism by benextramine at α_{2A} -adrenoceptors *via* a G_i -mediated effect only, it was important to investigate whether similar non-specific antagonism by benextramine can be observed *via* a G_s protein-mediated effect, but with the same receptors and effector (adenylyl cyclase). It has been shown before that after pertussis toxin (PTX) treatment of α_{2A} -H cells, the α_{2A} -adrenoceptors couple to G_s proteins to stimulate adenylyl cyclase activity (Brink et al., 2000). Figure 4–3 displays concentration-effect curves of UK 14,304 in PTX treated whole α_{2A} -H cells after pre-treatment with 0 or 100 μ M

benextramine plus 0 M (Figure 4–3A) or 10 μ M yohimbine (Figure 4–3B) for 20 minutes at 37°C. Without protection of the α_{2A} -adrenoceptors (Figure 4–3A), 100 μ M benextramine abolished effect ($E_{\max}$ for $a_1 = 100 \pm 13.1\%$ and $E_{\max}$ for $a_2 = -3.6 \pm 7.5\%$, $n = 3$, $p < 0.01$). When the α_{2A} -adrenoceptors were protected by 10 μ M yohimbine, the G_s -mediated effect was only partially inhibited ($E_{\max}$ of curve $b_1 = 100 \pm 18.9\%$ and $E_{\max}$ of curve $b_2 = 53.0 \pm 5.8\%$, $n = 3$, $p < 0.05$) and the EC_{50} value remained unchanged.

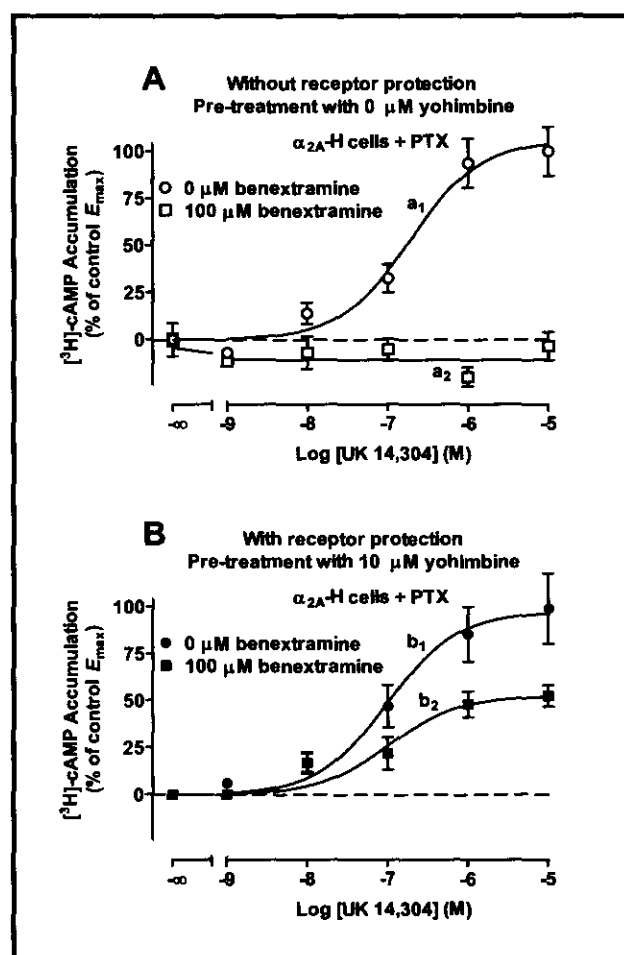


Figure 4–3: Semilogarithmic concentration-effect curves of UK 14,304 in α_{2A} -H cells treated with pertussis toxin observed G_s -mediated effects, measuring whole-cell [^{3} H]-cAMP accumulation. The α_{2A} -H cells were pre-treated with benextramine (0 or 100 μ M, 20 minutes) plus (A) 0 M yohimbine, or (B) 10 μ M yohimbine to protect α_{2A} -adrenoceptors. The data are represented as the mean $\pm$ S.E.M and expressed as percentage of the control $E_{\max}$ of curve a_1 . Data represent the average of triplicate observations of three experiments ($n = 3$). Concentration-effect curves are non-linear least square fits.

4.3.4 Agonist-induced, G_q -mediated [^{3} H]-IP_x accumulation in SH-SY5Y- and 5HT_{2A}-SH-SY5Y cells

We investigated whether benextramine pre-treatment would antagonize the effect in a signal-transduction system with a receptor type, G protein type and effector totally different from those in previous observations. In SH-SY5Y cells mACh receptors signal through G_q proteins to activate phospholipase C. Likewise in 5HT_{2A}-SH-SY5Y cells 5HT_{2A}-receptors signal through G_q proteins to activate phospholipase C. *Figure 4-4A* depicts concentration-effect curves of methacholine in SH-SY5Y cells and *Figure 4-4B* of serotonin in 5HT_{2A}-SH-SY5Y cells, measuring agonist-stimulated [^{3} H]-IP_x accumulation, after pre-treatment of the cells with 0 or 100 μ M benextramine for 20 minutes at 37°C.

In SH-SY5Y cells (*Figure 4-4A*) benextramine partially suppressed the methacholine-mediated effect ($E_{\max}$ values were $100 \pm 17.2\%$ for curve a₁ and $25.2 \pm 8.6\%$ for curve a₂, $n = 3$, $p < 0.05$). The EC_{50} value, however, remained unchanged. Likewise, in 5HT_{2A}-SH-SY5Y cells (*Figure 4-4B*) benextramine partially suppressed the serotonin-mediated effect ($E_{\max}$ values were $100 \pm 23.2\%$ for curve b₁ and $34.7 \pm 8.3\%$ for curve b₂, $n = 3$, $p < 0.05$). Again the EC_{50} value remained unchanged.

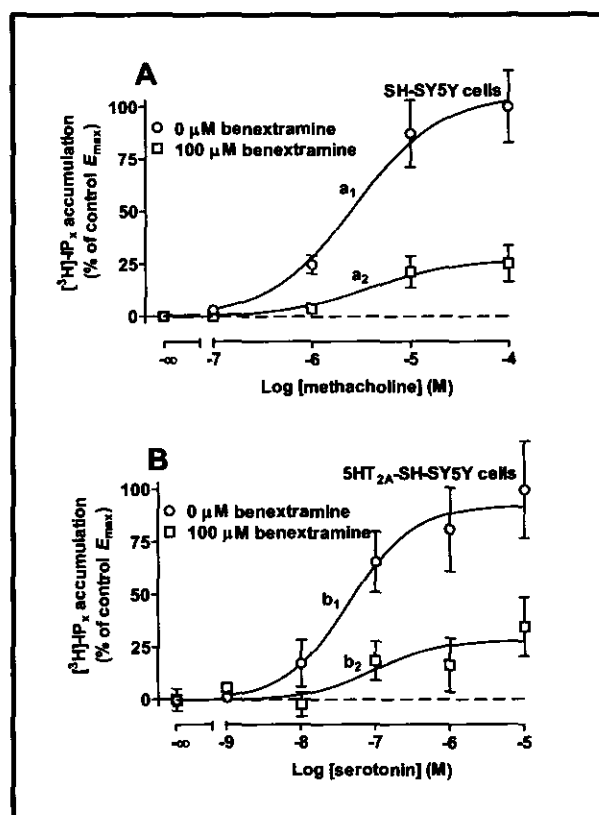


Figure 4-4: Semilogarithmic concentration-effect curves of (A) methacholine in SH-SY5Y cells, and (B) serotonin in 5HT_{2A}-SH-SY5Y cells. The cells were pre-treated with benextramine (0 or 100 μM, 20 minutes), whereafter whole-cell total $[^3H]$ -IP_x accumulation was measured with increasing concentrations agonist. The data are represented as mean $\pm$ S.E.M. and curves a₁ and a₂ are expressed as percentage of the E_{max} of curve a₁, while curves b₁ and b₂ are expressed as percentage of the E_{max} of curve b₁. Data represent the average of triplicate observations of three experiments ($n = 3$). The curves are non-linear least square fits.

4.3.5 Binding data for $[^3H]$ -4-DAMP at mACh receptors and $[^3H]$ -ketanserin at 5HT_{2A}-receptors

Since benextramine inhibits the $[^3H]$ -IP_x accumulation in both SH-SY5Y and 5HT_{2A}-SH-SY5Y cells (Figure 4-4), it was important to determine whether this inhibition could be ascribed to a reduction in receptor number, or whether non-specific antagonism is displayed by benextramine.

After pre-treatment of SH-SY5Y whole cells with 0 M or 100 μM benextramine for 20 minutes at 37°C, plus 0 M or 10 μM atropine ($>1,000 \times K_i$ value, to protect mACh receptors from binding to benextramine), radioligand binding assays were conducted to determine the relative number of receptors. Figure 4-5A and Figure 4-5B depict the specific binding of $[^3H]$ -4-DAMP to mACh receptors in SH-SY5Y cells after pre-

treatment with 0 M or 100 μ M benextramine. In the absence of receptor protection by atropine (*Figure 4–5A*), benextramine pre-treatment significantly reduced the specific binding of [^{3} H]-4-DAMP from $100 \pm 4.4\%$ (bar a_1) to $35.6 \pm 3.3\%$ (bar a_2) ($n = 3$, $p < 0.001$). However, after pre-treatment with 10 μ M atropine to protect the mACh receptors from benextramine (*Figure 4–5B*), the specific binding was also significantly decreased from $76.8 \pm 3.2\%$ (bar b_1) to $42.3 \pm 2.2\%$ (bar b_2) ($n = 3$, $p < 0.001$).

Likewise, after pre-treatment of 5HT_{2A}-SH-SY5Y whole cells with 0 M or 100 μ M benextramine for 20 minutes at 37°C, plus 0 M or 10 μ M ritanserin ($>1,000 \times K_i$ value, to protect 5HT_{2A}-receptors from binding to benextramine), radioligand binding assays were conducted to determine the relative number of receptors. *Figure 4–5C* and *Figure 4–5D* depict the specific binding of [^{3} H]-ketanserin to 5HT_{2A}-receptors in 5HT_{2A}-SH-SY5Y cells after pre-treatment with 0 M or 100 μ M benextramine. In the absence of receptor protection by ketanserin (*Figure 4–5C*), benextramine pre-treatment significantly reduced the specific binding of [^{3} H]-ketanserin from $100 \pm 12.3\%$ (bar c_1) to $31.1 \pm 4.9\%$ (bar c_2) ($n = 4$, $p < 0.01$). However, after pre-treatment with 10 μ M atropine to protect the mACh receptors from benextramine (*Figure 4–5D*), the specific binding was also significantly decreased from $85.4 \pm 19.3\%$ (bar d_1) to $30.0 \pm 6.0\%$ (bar d_2) ($n = 4$, $p < 0.05$).

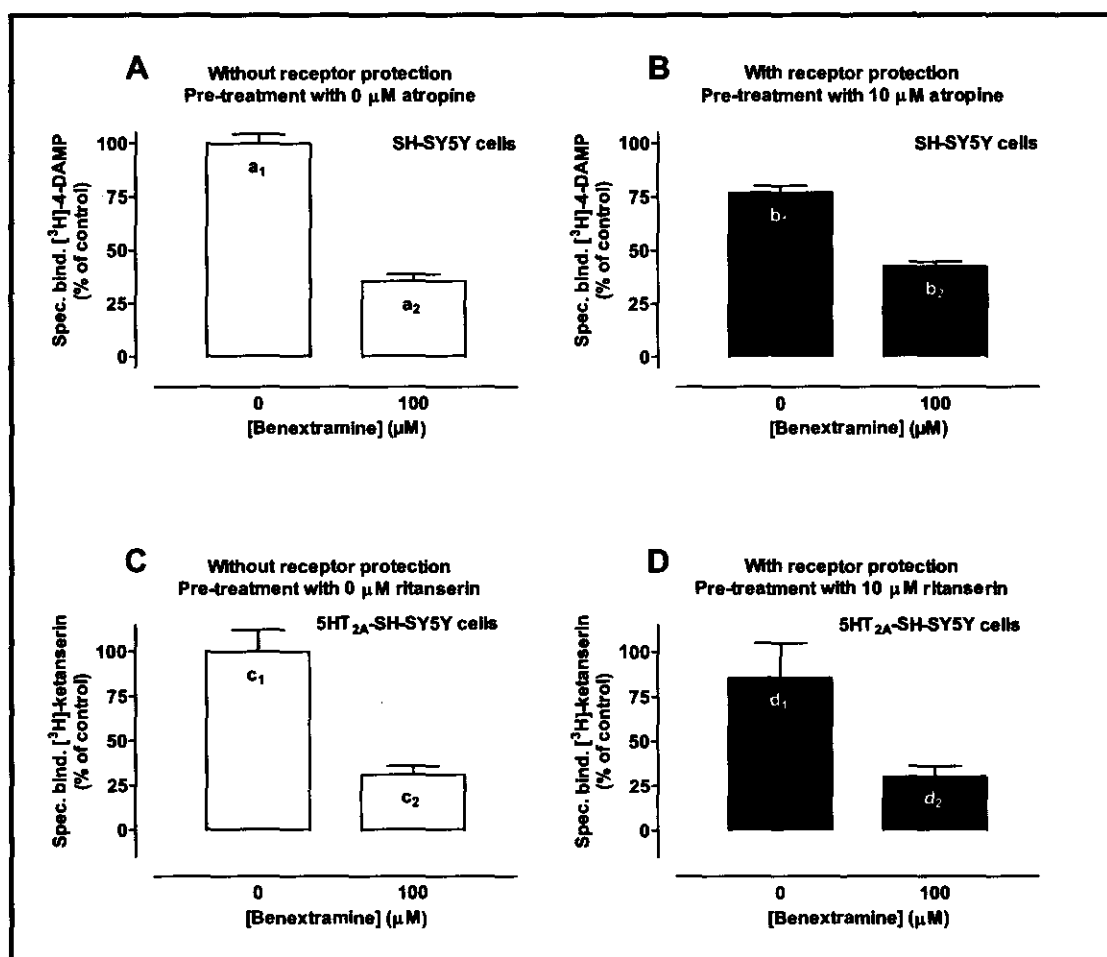


Figure 4–5: Specific binding of (A & B) 5 nM [^{3} H]-4-DAMP in SH-SY5Y cells, or (C & D) 5 nM [^{3} H]-ketanserin in 5HT $_{2A}$ -SH-SY5Y cells. The cells were pre-treated with benextramine (0 or 100 μ M, 20 minutes) and (A) 0 M atropine, or (B) 10 μ M atropine to protect mACh receptors, and (C) 0 M ritanserin, or (D) 10 μ M ritanserin to protect 5HT $_{2A}$ receptors. Thereafter, whole-cell specific binding was determined. The bar graphs represent the mean specific binding $\pm$ S.E.M. and are expressed as percent of control samples without benextramine and atropine or ritanserin. Data represent the average of triplicate observations of three experiments ($n = 3$) in (A & B) and four experiments ($n = 4$) in (C & D).

4.4 Discussion

Recent observations in our laboratory (see Chapter 3) suggest that, besides its known action as an irreversible antagonist at α_{2A} -adrenoceptors by binding these receptors irreversibly, benextramine also displays significant irreversible non-specific antagonism. The non-specific antagonism at α_{2A} -adrenoceptors was evident when measuring agonist-induced reduction in [^{3} H]-cAMP accumulation in whole cells, an effect resulting from α_{2A} -adrenoceptor-mediated activation of G_i proteins, whereby adenylyl cyclase activity is inhibited. In the present study, we have extended our

investigation of the mechanism whereby benextramine displays its irreversible non-specific antagonism, and in particular to identify where in the signal transduction system the non-specific antagonism is induced. In addition, we have investigated the effect of benextramine on other G protein-mediated signal transduction systems.

4.4.1 The non-specific irreversible antagonism by benextramine at α_{2A} -adrenoceptors can be explained by the inhibition of receptor and/or G_i protein function

From the results represented in *Figure 4-1* it can be seen that the irreversible antagonism by benextramine in the pre-treatment cannot be prevented by the simultaneous protection of the α_{2A} -adrenoceptors with a high concentration of the reversible competitive antagonist yohimbine. After the pre-treatment and membrane preparation, the measured [^{35} S]-GTP γ S binding in cell membranes results from the UK 14,304-mediated activation of α_{2A} -adrenoceptors and the consequent coupling to and activation of endogenous G proteins. Since [^{35} S]-GTP γ S binding was still abolished in membranes where the α_{2A} -adrenoceptors were protected from binding to benextramine during the pre-treatment, these results confirm the previously observed non-specific antagonism by benextramine (see *Chapter 3*). Furthermore, the non-specific antagonism is now observed further downstream in the signal transduction system of the α_{2A} -adrenoceptor and the data suggest that the non-specific antagonism by benextramine results from irreversible binding at the level of the α_{2A} -adrenoceptors and/or G protein, by inhibiting either the receptor G protein coupling, G protein activation or [^{35} S]-GTP γ S binding capacity of the G protein. Since GTP γ S is a non-hydrolysable analogue of the endogenous GTP, it does not appear likely that altered GAP function (enhanced GTP hydrolysis with G protein inactivation) is the cause. α_{2A} -adrenoceptors have previously been shown to couple with high efficiency to pertussis toxin (PTX)-sensitive G_i proteins (Chabre et al., 1994; Eason et al., 1992), which is present in the CHO-K1 cells used (Eason et al., 1992), but with much lower efficiency to G_s and G_q proteins (Brink et al., 2000; Chabre et al., 1994; Wade et al., 1999). Therefore, it can be reasonably assumed that the observed [^{35} S]-GTP γ S binding results from predominantly G_i protein activation. This is confirmed by unpublished

data from the laboratory of Dr Rick Neubig (Departments of Pharmacology and Internal Medicine (Division of Hypertension), University of Michigan, Ann Arbor, MI, U.S.A.) that the α_{2A} -adrenoceptor-stimulated [^{35} S]-GTP γ S binding to α_{2A} -H cell membranes is completely abolished after PTX pre-treatment of the cells prior to membrane preparation.

4.4.2 Non-specific antagonism by benextramine does not involve direct inhibition of [^{35} S]-GTP γ S binding

From the results in *Figure 4-2* it follows that the pre-treatment of $G_o\alpha$ with benextramine at a relatively high concentration and at two extreme and distinct temperatures and incubation times does not inhibit the constitutive binding of [^{35} S]-GTP γ S to $G_o\alpha$. $G_o\alpha$ protein is commercially available as a purified and highly stable GTP-binding protein. Both $G_o\alpha$ and $G_i\alpha$ are PTX-sensitive, but cholera toxin-insensitive and therefore belong to the same G protein family ($G_{i/o}$ family) (Sternweis and Robishaw, 1984). In addition, $G_o\alpha$ and $G_i\alpha$ display similar agonist-mediated α_{2A} -adrenoceptor signaling properties, as measured by [^{35} S]-GTP γ S binding and as observed for a series of agonists (Yang and Lanier, 1999). Due to the above-mentioned comparable signaling characteristics of $G_o\alpha$ and $G_i\alpha$ and the results presented in *Figure 4-2*, it is reasonable to assume that benextramine will also not modulate the binding capacity of $G_i\alpha$ for [^{35} S]-GTP γ S. We therefore propose that the observed non-specific antagonism by benextramine at α_{2A} -adrenoceptors, as presented in *Figure 4-1*, most likely results from an inhibition of the α_{2A} -adrenoceptor- $G_i\alpha$ protein coupling and not from an inhibition of the GTP binding properties of $G_i\alpha$ as such.

4.4.3 Non-specific antagonism by benextramine is also evident when measuring a G_s -mediated effect from agonist-mediated stimulation of α_{2A} -adrenoceptors

It has been shown that α_{2A} -adrenoceptors also signal through G_s proteins to activate adenylyl cyclase, although the coupling efficiency to G_s was reported to be

approximately 1,000 times lower (Chabre et al., 1994). This is evident in systems with a high α_{2A} -adrenoceptor concentration, after treatment with PTX to inhibit G_i proteins, by measuring increased cAMP accumulation resulting from the G_s -mediated stimulation of adenylate cyclase (Wade et al., 1999). From the results in *Figure 4–3* it is evident that benextramine also displays its non-specific antagonism at α_{2A} -adrenoceptors when a UK 14,304-stimulated, G_s -mediated increase in [^{3} H]-cAMP accumulation is measured. As mentioned above, previous studies in our laboratory (see *Chapter 3*) suggested non-specific antagonism at α_{2A} -adrenoceptors when a UK 14,304-stimulated, G_i -mediated decrease in [^{3} H]-cAMP accumulation was measured. When taken into account that the non-specific antagonism by benextramine is displayed most likely at the receptor G_i protein level and that the non-specific antagonism is seen also when the α_{2A} -adrenoceptors signal through G_s proteins, it can be proposed that benextramine most likely binds to either a non-specific binding site on the α_{2A} -adrenoceptor macromolecule or a common binding site on the G_i and G_s proteins that interfere with receptor G protein coupling. However, when comparing *Figure 4–3B* with *Figure 3–3D* of previous studies in our laboratory, it appears that the non-specific antagonism of benextramine at α_{2A} -adrenoceptors when measuring UK 14,304-stimulated, G_s -mediated effects is substantially less than for measuring G_i -mediated effects. These results suggest that the irreversible non-specific antagonism by benextramine is dependent on the G protein type involved through which the GPCR signals. It is also known that there are differences in the distinct basic residues of the α_{2A} -AR that mediate G_i and G_s activation, and that the α_{2A} -AR couples preferentially to G_i (Wade *et al.*, 1999). In addition, the suggested mechanism of action of benextramine is to form a disulphide bridge between itself and a target thiol group at the GPCR (Brasili *et al.*, 1980; 1986; Melchiorre & Gallucci, 1983; Melchiorre *et al.*, 1979), thereby affecting the stereochemical properties of cysteine amino acid residues and probably disrupting the coupling of the α_{2A} -AR to G_i more than to G_s .

4.4.4 Non-specific antagonism by benextramine is evident when measuring a G_q -mediated effect from agonist-mediated stimulation of both mACh receptors and 5HT_{2A}-receptors

Both mACh receptors (Sorensen et al., 1999) and 5HT_{2A} receptors (Berridge, 1993) have been shown to signal through PTX-insensitive G_q proteins and thereby activate phospholipase C (PLC) to promote the synthesis of the second messengers inositol (1,4,5)-trisphosphate (IP₃) and diacylglycerol. In addition, we have observed that an increase in IP_x accumulation, as induced by agonist-mediated stimulation of mACh receptors, is abolished by the PLC inhibitor U-73122 (Bleasdale and Fisher, 1993) and also that this mACh receptor-mediated IP_x accumulation is PTX insensitive. From the results in *Figure 4-4A*, it can be seen that benextramine irreversibly inhibits the methacholine-induced G_q -mediated signaling of mACh receptors in SH-SY5Y cells (significant reduction in E_{max}). The corresponding benextramine-induced decrease in the relative receptor number is depicted in *Figure 4-5A*. However, *Figure 4-5B* shows that atropine at a relative high concentration of 10 μ M ($>1,000 \times K_D$ value) was not able to protect the mACh receptors against inactivation by benextramine. These results suggest that benextramine acts *via* a non-specific binding site and that it displays irreversible non-specific antagonism at mACh receptors. In contrast, Benfey et al. (1980) suggested that the antagonism by benextramine at mACh receptors was reversible when measuring smooth muscle contraction in isolated guinea-pig atrium and ileum. Species and tissue differences between our results and the results of Benfey and coworkers may explain the apparent contradiction.

From the results in *Figure 4-4B*, it can be seen that benextramine irreversibly inhibits the serotonin-induced, G_q -mediated signaling of 5HT_{2A}-receptors in 5HT_{2A}-SH-SY5Y cells (significant reduction in E_{max}). The corresponding benextramine-induced decrease in the relative receptor number is depicted in *Figure 4-5C*. However, *Figure 4-5D* shows that ritanserin at a relative high concentration of 10 μ M ($>1,000 \times K_D$ value) was not able to protect the 5HT_{2A}-receptors against inactivation by benextramine. As with mACh receptors, these results suggest that benextramine acts *via* a non-specific binding site and that it displays irreversible non-specific antagonism at 5HT_{2A} receptors. Stanton and Beer (1997) indicated that benextramine antagonized

the effect of serotonin at 5HT_{1A}-receptors, and we provide data that benextramine acts as a non-specific irreversible antagonist also at 5HT_{2A}-receptors.

The above-mentioned data for both mACh and 5HT_{2A} receptors suggest that benextramine reduces the number of receptors available for binding to their respective ligands, as can be deduced from the observations of decreased radioligand binding. This could NOT be prevented by protecting the receptors with a high concentration of an appropriate reversible competitive antagonist. If the mACh- and 5HT_{2A}-receptors were not sufficiently protected by the high concentrations of their respective reversible competitive antagonists, this could have explained the observed results. However, this is highly unlikely in the light of the high concentrations of more than $1,000 \times$ the K_D values of the respective reversible competitive antagonists that were used, a similar concentration to that proved to be effective for yohimbine at α_{2A} -adrenoceptors. We therefore propose that the binding sites of benextramine at mACh and 5HT_{2A} receptors are non-specific (i.e. at a different location on the receptor macromolecule than the specific binding site). These non-specific binding sites are likely to be allosteric in the sense that they render the specific binding sites on the receptor macromolecules inactive (unable to bind to their ligands).

4.4.5 Final conclusions

We provide here evidence for the hypothesis that the irreversible antagonist benextramine inhibits different agonist-induced effects at several GPCRs that signal through different G protein types in different pharmacological systems, presumably by binding to non-specific binding sites at the various receptor types. At α_{2A} -adrenoceptors, data suggest that benextramine antagonizes G_i and to a lesser degree G_s -mediated effects and this is done presumably by inhibiting receptor G protein coupling. This suggests that the differences in the degree of irreversible non-specific antagonism are related to the type of G protein involved. However, at both mACh receptors and 5HT_{2A} receptors, data suggest that benextramine binds to an allosteric binding site on the receptor macromolecule that inhibits the binding of ligands to the agonist binding site and thereby prevents agonist-induced effect, rather than modulating downstream G protein activation. Importantly, the current data however, does not exclude the

possibility that receptor G protein coupling is also inhibited at mACh receptors and 5HT_{2A}-receptors, as was suggested for α_{2A} -adrenoceptors.

The current observations need further investigation to clarify the exact mechanisms of action of benextramine at α -adrenoceptors, mACh receptors and 5HT_{2A}-receptors. Benextramine may prove to be a useful experimental tool in investigating the signaling mechanisms of G protein-coupled receptors. In addition, the data suggest that irreversible antagonists, in particular benextramine, may display irreversible non-specific antagonism through different mechanisms in different biological systems and this may influence our interpretation of data obtained with these drugs.

Acknowledgements

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Summary and Conclusions**Chapter
5**

Concerns have been raised that irreversible antagonists may display irreversible non-specific antagonism to modulate the relationship between pharmacological stimulus and effect or to affect the binding affinity of ligands for their specific receptors. This study investigated whether a selection of classical irreversible antagonists, namely phenoxybenzamine, benextramine and 4-DAMP mustard, display irreversible non-specific antagonism. New insight was gained into the mechanisms of action of these irreversible antagonists, and in particular of the subcellular mechanism of action of benextramine at different G protein-coupled receptor (GPCR) types and signal transduction systems.

5.1 Summary

Complementary functional and radioligand binding data that describe the non-specific mechanisms of antagonism by phenoxybenzamine, benextramine and 4-DAMP mustard were obtained from various cell lines expressing different types of GPCRs.

Firstly, it was observed in α_{2A} -H and α_{2A} -L cells¹ by measuring the agonist-induced inhibition of [³H]-cAMP accumulation, that the irreversible antagonist benextramine displays significant irreversible specific and non-specific antagonism at α_{2A} -adrenoceptors (α_{2A} -ARs) when employed at a concentration of 10 and 100 μ M for 20 minutes. The non-specific component of the antagonism is dependent on the concentration of benextramine and the incubation time of the cells. At 100 μ M for 20 minutes the non-specific antagonism was sufficient to cause rightward shift of about 140-fold in the concentration-effect curve of UK 14,304. This could not be prevented by protecting the α_{2A} -adrenoceptors with a sufficiently high concentration (10 μ M) of

¹ Chinese hamster ovary (CHO-K1) cells transfected to express porcine α_{2A} -adrenoceptors at higher (α_{2A} -H) or lower (α_{2A} -L) numbers (see *Section 3.2.1*).

the reversible competitive antagonist yohimbine. Phenoxybenzamine employed at 100 μM for 20 minutes, however, displayed a relatively small component of irreversible non-specific antagonism when compared to the observed irreversible non-specific antagonism by benextramine. In addition, results suggest that the mechanism of irreversible non-specific antagonism by phenoxybenzamine and benextramine is not due to altered affinity of the receptor for the α_{2A} -AR agonist UK 14,304 (i.e. not allosteric antagonism), but that it is rather related to an inhibition of the signal transduction of the α_{2A} -AR.

$[^{35}\text{S}]$ -GTP γ S binding studies in α_{2A} -H membranes showed inhibition of UK 14,304-mediated response after benextramine pre-treatment, even when the α_{2A} -adrenoceptors were protected by 10 μM yohimbine. These results suggest that the observed irreversible non-specific mechanism of antagonism by benextramine at α_{2A} -ARs is induced by irreversible binding of benextramine at the level of the receptor or G protein. In addition, it was found that benextramine pre-treatment did not modulate the constitutive binding of $[^{35}\text{S}]$ -GTP γ S to G_o protein, a G protein with similar α_{2A} -adrenoceptors-mediated signalling properties than G_i . It was therefore concluded that benextramine displays its irreversible non-specific antagonism at α_{2A} -ARs by modulating receptor signalling, most likely by inhibiting the α_{2A} -AR- G_i protein coupling.

In addition, benextramine displays significant irreversible specific and non-specific antagonism at α_{2A} -ARs when signalling through G_s proteins. This property of benextramine became apparent when α_{2A} -H cells were pre-treated with pertussis toxin (to inhibit the G_i -mediated inhibition of adenylate cyclase) and the agonist-induced G_s -mediated stimulation of $[^3\text{H}]$ -cAMP accumulation was measured. The non-specific component of irreversible antagonism at 100 μM for 20 minutes was sufficient to cause a significant reduction in the maximal effect of the concentration-effect curve of UK 14,304, when the α_{2A} -ARs were protected by 10 μM yohimbine.

Pre-treatment with benextramine reduced the muscarinic acetylcholine receptor (mACh-R) or 5HT $_{2A}$ -serotonin receptor (5HT $_{2A}$ -R) induced G_q -mediated accumulation

of [^3H]-IP $_x$ in SH-SY5Y² or 5HT $_{2A}$ -SH-SY5Y³ cells, respectively. However, the reduction in specific receptor numbers (measured with radioligand binding assays) could not be prevented by protecting the mACh-Rs in SH-SY5Y cells or 5HT $_{2A}$ -Rs in 5HT $_{2A}$ -SH-SY5Y cells respectively with a sufficiently high concentration (10 μM) of the reversible antagonists atropine or ritanserin.

Importantly, there appear to be clear differences among the mechanisms of antagonism by benextramine on the 4 different signalling mechanisms that were investigated in this study (α_{2A} -AR-G $_i$; α_{2A} -AR-G $_s$; mACh-R-G $_q$; and 5HT $_{2A}$ -G $_q$). Regarding the agonist-mediated signalling of α_{2A} -ARs through G $_i$ and G $_s$, it was observed that the irreversible non-specific antagonism by benextramine is substantially less pronounced for G $_s$ than for G $_i$. These results suggest that the irreversible non-specific antagonism by benextramine is dependent on the G protein involved through which the GPCR signals. It is also known that there are differences in the distinct basic residues of the α_{2A} -AR that mediate G $_i$ and G $_s$ activation, and that the α_{2A} -AR couples preferentially to G $_i$. In addition, the suggested mechanism of action of benextramine is to form a disulphide bridge between itself and a target thiol group at the GPCR, thereby affecting the stereochemical properties of cysteine amino acid residues and probably disrupting the coupling of the α_{2A} -AR to G $_i$ more than to G $_s$. Therefore, α_{2A} -AR-G $_i$ signalling is inhibited since the α_{2A} -AR is unable to couple efficiently to G $_i$, and that might account for the observations that benextramine does not inhibit the constitutive binding of [^{35}S]-GTP γS to purified G $_{\alpha}$ and thereby the G protein activity. However, the irreversible antagonistic actions of benextramine observed with mAChRs and 5HT $_{2A}$ -Rs that signal through G $_q$ are distinct from those observed with α_{2A} -ARs that signal through G $_i$ and G $_s$. Results suggest that benextramine irreversibly inhibits the binding of ligands instead of modulating the downstream activation of G $_q$.

Lastly, by measuring agonist-induced, G $_q$ -mediated [^3H]-IP $_x$ accumulation in SH-SY5Y cells, it was observed that the irreversible antagonist 4-DAMP mustard displays irreversible specific but not irreversible non-specific antagonism at muscarinic acetylcholine receptors when it was employed at 100 nM for 20 minutes. However,

² Human neuroblastoma cells that endogenously express muscarinic acetylcholine receptors (see Section 3.2.1).

³ Human neuroblastoma (SH-SY5Y) cells transfected to express human 5HT $_{2A}$ -serotonin receptors (see Section 4.2.5).

when 4-DAMP mustard was employed at a concentration of 100 nM and the incubation time increased from 20 minutes to 60 minutes, significant irreversible non-specific antagonism was observed. This observation illustrated the influence of experimental conditions such incubation time on the non-specific antagonistic behaviour of 4-DAMP mustard.

5.2 Final conclusions

The classical irreversible antagonists phenoxybenzamine, benextramine and 4-DAMP mustard display irreversible non-specific antagonism at typical experimental conditions. These findings support the possibility that other irreversible antagonists employed in the experimental pharmacology could also display a component of irreversible non-specific antagonism. This could influence the interpretation of data obtained after pre-treatment with an irreversible antagonist. Therefore, when it is intended to employ an irreversible antagonist to inactivate specific receptors without modulating the stimulus-effect relationship, it is important to establish optimal experimental conditions to minimise a possible nescient component of non-specific irreversible antagonism and thereby its influence on the relationship between stimulus and pharmacological effect. Typically, the optimal experimental conditions should be chosen where the irreversible antagonist sufficiently reduces the specific receptor number without significant non-specific antagonism. Such conditions should be confirmed by a combination of appropriate functional and radioligand-binding data.

Finally, the irreversible antagonist benextramine may prove to be a useful tool in studying agonist-induced GPCR signalling through other G protein types in various pharmacological systems, especially due to its likely inhibition of the α_{2A} -AR- G_i protein coupling. It may also be a useful tool in studying structural/conformational determinants of receptor macromolecules (e.g. of mACh-Rs or 5HT_{2A}-Rs) for its ligand-binding properties. However, an even better understanding of the inhibitory action of benextramine at these receptors may be needed before data obtained from such studies will contribute to our current understanding of GPCR signal transduction or GPCR ligand-binding.

***Recent Advances in Drug
Action and Therapeutics:
Relevance of Novel Concepts in
G Protein-coupled Receptor
and Signal Transduction
Pharmacology***

**Appendix
A**

Educational Review Paper

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Abstract

During especially the past two decades many discoveries in biological sciences, and in particular at molecular and genetic level, have greatly impacted on our knowledge and understanding of drug action. The multitude of novel concepts in molecular receptor and subcellular signal transduction pharmacology have helped us to not only better understand and improve current therapeutic strategies, but also to develop new drugs and strategies. Furthermore, many exciting new drugs acting *via* novel pharmacological mechanisms are expected to be in clinical use in the not too distant future. In this educational review, these concepts are explained and their relevance illustrated by examples of drugs used commonly in the clinical setting. Special reference is given to the pharmacology of G protein-coupled receptors, since more than 40% of all drugs on the market act *via* this large family of receptors. This educational review also addresses the concepts of full and partial agonism, neutral antagonism, inverse agonism and protean and ligand-selective agonism. Moreover, the mechanisms whereby receptor signalling (and eventually effect to drugs) is fine-tuned are explained. In this regard, concepts such as receptor promiscuity, agonist-directed trafficking of receptor signalling, receptor trafficking, receptor "cross-talk" and regulators of G protein signalling (RGSs) are discussed, from theory to proposed therapeutic implications. It is concluded that the understanding of molecular receptor and signal transduction pharmacology will enable clinicians to improve their effective implementation of current and future pharmacotherapy, ultimately enhancing the quality of life of their patients.

A.1 Introduction

We are all aware of the positive and negative side of drugs, of the ways in which they can enhance or decrease our quality of life, or even save or take lives. Whenever the potential therapeutic benefit of a drug is considered to outweigh its potential hazards, optimal drug selection needs to be made and sound pharmacotherapy becomes the ideal of every good clinical therapist. But rational and optimal pharmacotherapy has to be based on strong pillars of appropriate knowledge, skills and values. We need appropriate **knowledge** of basic pharmacology and evidence-based medicine, adequate **skills** to diagnose, interpret and synthesise creative solutions and applicable **values** to realise and respect the fact that it is all about a human being with his/her own preferences and individual criteria for evaluating quality of life. In this review we will emphasise the importance of basic pharmacology, in particular of receptor pharmacology and subcellular signal transduction. This knowledge is not only important in the understanding of current therapeutics, but also to create innovative strategies when there is no clear standard solution and also to be geared to understand the medicine of tomorrow.

In this regard the General Medical Council in Great Britain recently called for an investigation into the knowledge and skills required to ensure current and future standards for medical practitioners. By using appropriate questionnaires Mucklow (2002)¹ obtained the opinion of representative specialists in clinical pharmacology and therapeutics on this issue. Interestingly, but maybe not totally surprisingly, Mucklow found that knowledge of basic molecular receptor pharmacology and signal transduction mechanisms were rated as essential by invariably 100% of the respective representative panels.

There are many examples of how knowledge of basic molecular and signal transduction pharmacology can be of value in the clinical setting. In the 1980's the introduction of the selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, into clinical medicine was heralded as the first class of antidepressants with a selective action on a key neurobiological target in depression, namely serotonin. These drugs were the antithesis of their "dirtier" predecessors, the tricyclic antidepressants (TCAs), which acted on a wide range of neurotransmitter receptor systems not necessarily

linked to the neuropathology of depression. Consequently, the SSRIs were marketed as pharmacologically "pure" antidepressants with a minimal risk of adverse effects related to actions at "unwanted" (untargeted) receptors in the brain and elsewhere. These predictions and marketing strategy was only in part true. As the SSRIs realised greater popularity, evidence for rare yet troublesome adverse events began to appear. Most notable were symptoms resembling that evoked by typical neuroleptic agents, such as dystonia, extrapyramidal effects and the potentially fatal serotonin syndrome^{2,3,4,5}. This suggested an action in the basal ganglia, probably involving attenuation of neuro-motor dopamine pathways, which was not initially expected for SSRIs. The SSRIs act by inhibiting synaptic reuptake of extracellular serotonin (5HT), thereby stimulating 5HT pathways in the brain. However, 5HT-receptors on dopamine projections in the striatum are also activated, resulting in a suppression of dopamine synthesis and dopamine release⁶. This action produces a hypodopaminergic state, which has been linked to the aforementioned side-effects⁷. This insight into the mechanism of action of the SSRIs provides just one example of how extracellular receptor selectivity may translate into intercellular receptor promiscuity. This example describes how one receptor type may communicate with another within the same cell, or across different cells. These communication mechanisms between receptors and other signal modulating mechanisms are the focus of the current review.

Molecular receptor and signal transduction pharmacology has advanced significantly in the second half of the previous century and particularly during the last two decades, with many emerging new and exiting concepts and models of drug action. There are many exciting new drugs acting *via* novel pharmacological mechanisms that are expected to be in clinical use in the not so distant future. More than 40% of all marketed drugs display activity *via* G protein-coupled receptors and with current bio-informatics it is expected that several new drug targets related to the G protein-coupled receptors will be discovered and their physiological and clinical significance explored in future⁸. The clinician needs to be equipped and prepared to understand the actions of these drugs and the rationale for their therapeutic uses. This review aims to assist clinicians to gain a deeper understanding of drug action and the latest developments in the basic pharmacological sciences and its relationship to clinical therapeutics. It will also point out the likely future trends in drug treatments, with special emphasis on the

so-called **G protein-coupled receptors** (a special family of drug receptors that will be explained in this review) and their **signal transduction mechanisms**.

A.2 Important classical and novel concepts

In order to understand the therapeutic implications of the pharmacology of G protein-coupled receptors and their signal transduction systems one has to gain some knowledge of key concepts and terminology.

This review addresses the current understanding of G proteins, G protein-coupled receptors, full agonists, partial agonists, neutral antagonists, inverse agonists and even protean and ligand-selective agonists. Moreover, concepts such as receptor promiscuity (receptor heterogeneity), agonist-directed trafficking of receptor signalling, receptor trafficking, receptor desensitisation, receptor down regulation and recycling of receptors, receptor "cross-talk" and regulators of G protein signalling (RGSs) will also be discussed, from theory to proposed therapeutic implications.

A.2.1 Pharmacological receptors

Many agents such as neurotransmitters, hormones and drugs (and even light photons and odorants) transfer their signals to cells by interaction with a membrane receptor at the cell surface⁹, upon which the cell responds with a series of intracellular events (intermediate effects collectively referred to as the signal transduction system) that eventually lead to altered cellular function(s). The resulting altered cellular function may in turn affect the function of a tissue or organ or a system of the body. To understand new drug developments, a deeper understanding of this signalling process is required, starting from the drug-receptor interaction to the final cellular effect. Several receptor families can be distinguished, of which the ligand-gated ion channels, receptor protein kinases, G protein-coupled receptors and transcription factor receptors are typical examples¹⁰:

- **Ligand-gated ion channel receptors:** A subgroup of membrane receptors is proteins or protein complexes that form ion channels, known as ligand-gated ion channels (where the word "ligand" here refers to the binding transmitter or drug)¹⁰. Examples of ligand-gated ion channels include the **nicotinic**

acetylcholine receptors, **GABA_A**-receptors and **serotonin-3 (5HT₃-)** receptors. Binding of the ligand to the receptor opens ("gates") the ion channel to allow for the flow of particular ions across the membrane.

- **Receptor protein kinases:** The receptor protein kinases are receptor enzyme molecules involved in the transfer of extracellular signals to the intracellular domain, where the kinases phosphorylate effector proteins to alter their function¹⁰. A typical example is the **insulin** receptor.
- **G protein-coupled receptors:** The G protein-coupled receptors (commonly abbreviated GPCRs) are a large family of seven-transmembrane spanning receptors^{11,12}, amongst others the **adrenergic**, **dopaminergic**, **serotonergic**, **muscarinic** acetylcholine and **histaminergic** receptor types. The GPCRs are so named by their common ability to activate so called G proteins (guanine-nucleotide- or GTP-binding proteins), whereby they alter cell function. Being large protein molecules situated in the cell membrane (spanning the membrane seven times with their coils or helices), GPCRs are able to transfer the signal from a drug bound to the extracellular surface to the intracellular surface (see *Figure A-1*). This is achieved by a change in conformation (spatial orientation) to activate the G proteins at the intracellular surface of the membrane. The G protein then transfers the signal further down in a cascade of intracellular processes. One could refer to three components in this signalling process, namely the receptor (GPCR), the transducer (G protein) and the effector (e.g. adenylyl cyclase, phospholipase C, Ca²⁺ channels and K⁺ channels)¹⁰.
- **Transcription factor receptors:** Several hormones (e.g. **corticosteroidal** hormones, **thyroid** hormone, **vitamin D**, etc.) bind to intracellular DNA-binding proteins that act as receptors to regulate the transcription of particular genes, with subsequent regulation of protein synthesis¹⁰.

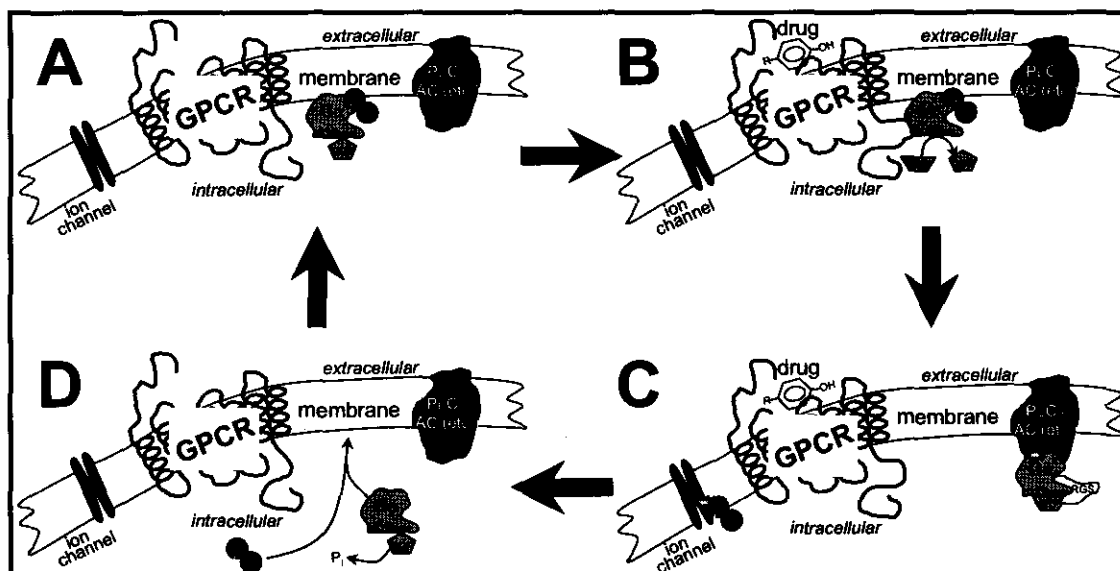


Figure A-1: A schematic representation of the G protein "activation/deactivation cycle", associated with the signalling mechanism of G protein-coupled receptors (GPCRs). Heterotrimeric G proteins consist of α - and $\beta\gamma$ -subunits. Assume a case of no significant constitutive receptor activity. **(A)** In the resting (inactive) state the GPCR is not coupled to the G protein. **(B)** As the agonist binds to the receptor, the equilibrium between the R and R* states is disturbed, so that a larger fraction of the GPCRs is in the R* conformation. The R* conformation couples efficiently with the G protein, leading to the exchange of GDP for GTP on the $G\alpha$ -subunit. **(C)** The $G\beta\gamma$ -subunit is released and both $G\alpha$ and $G\beta\gamma$ interact with their respective effectors to continue the transduction of the signal. **(D)** After hydrolysis of GTP to GDP on the $G\alpha$ -subunit (under influence of GTPase plus RGS) the $G\alpha$ and $G\beta\gamma$ -subunits reunite. The system returns to its original state as presented in **(A)** and is ready for the next GPCR-mediated activation. PLC = phospholipase C; AC = adenylyl cyclase; GPCR = G protein-coupled receptor; GDP = guanosine diphosphate; GTP = guanosine triphosphate.

A.2.2 G protein-coupled receptors

For the purpose of this review, the GPCRs and their function will be emphasised. A considerable degree of research has been focussed on the pharmacology and signal transduction mechanisms of GPCRs, to such an extent that we may expect future new drugs to modulate GPCR function in novel ways. GPCRs are involved in the regulation of an array of diverse physiological functions after activation by neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides and calcium ions^{12,13}. With an estimated 1% of the mammalian genome coding for GPCRs, thousands of GPCRs are expected to exist in the human body^{12,14}. Moreover, several GPCRs have now been discovered with their endogenous ligands still unknown. These GPCRs have been termed "orphan receptors". Orphan receptors are indeed viewed as

potential new drug targets and are currently being exploited for their potential in treating debilitating diseases¹⁵.

A.2.3 GPCR and theories of drug action

Current theories of GPCR function have greatly impacted our understanding of drug action and opened up new ways of searching for new drugs. The GPCR is a large protein that is in equilibrium between (presumably) several possible conformational states (spatial orientations)^{13,16,17,18}. Some of these conformational states are energetically more favourable than others. It is important to note that some of these conformations are assumed to be inactive (i.e. they do not activate G proteins) while others are active (i.e. they activate G proteins). We refer to models building on this concept as models of "multiple activation states of receptor activity". As illustrated in *Figure A–2*, the simplest theoretical model of these would assume only two conformations (two-state model), namely one inactive and one active conformation (usually designated the R and R* receptor states respectively)¹⁹. There is also a three-state model of receptor activation, with one inactive state (R) and two active states (R* and R**), where the one active state will couple to one type of G protein and the second to another type of G protein^{18,20}. The significance of these models for drug therapy will be discussed further below.

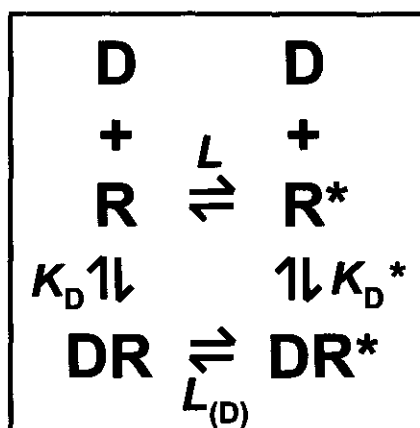


Figure A–2: A schematic representation of the two-state receptor model. R, R*, DR and DR* are in constant equilibrium, where D is the drug, R is the receptor in the inactive state, R* is the receptor in the active state, and DR and DR* are the respective drug-receptor complexes (drug-bound receptor). K_D , K_D^* , L and $L_{(D)}$ are kinetic constants describing the equilibrium between the respective states. In particular, K_D and K_D^* describe the affinity (binding power) of the drug for the receptor in its inactive and active states respectively.

Usually, in the two-state model, most of the GPCRs, when not bound to a drug, will exist in the inactive R conformation, with only a small fraction in the active R* conformation. However, some GPCRs have a significant fraction of the receptors in the R* conformation. The receptors in the R* conformation will give rise to a basal effect (also known as constitutive activity) of which the magnitude depends on the fraction of receptors in the R* conformation (i.e. the R:R* ratio). For any drug to influence the function of a receptor it has to be able to bind to the receptor, that is, it must foremost have **affinity** (binding power) for the receptor. When a drug has significant affinity for the receptor, it is predicted that drugs may bind selectively to either R or R* or with equal affinity for both R and R*. Receptor-binding drugs can therefore influence receptor function in one of three ways:

- **Full and partial agonists:** Some endogenous substances and drugs preferentially bind to R* and will then drive the equilibrium between R and R* towards more R*. This will increase receptor signalling and eventually pharmacological effect. These endogenous substances and drugs are known as **agonists**, such as *l*-noradrenalin, serotonin, dopamine and drugs such as dobutamine on β_1 -adrenergic receptors, isoproterenol on $\beta_{1/2}$ -adrenergic receptors, fenoldopam on D₁-receptors and clonidine on α_2 -adrenergic receptors and moxonidine on imidazoline I₁-receptors, to name but a few. The selectivity of agonists for R or R* may vary so that an agonist with great selectivity for R* over R will behave as a **full (strong) agonist** and one with only a small selectivity for R* over R, will behave as a **partial (weak) agonist** (see *Figure A-3*). Buspirone and oxymetazoline are typical examples of partial agonists on 5HT_{1A} and α_2 -adrenergic receptors, respectively. Several β -adrenoceptor blockers, e.g. pindolol and acebutalol, act as partial agonists at β -adrenoceptors and exhibit therefore intrinsic sympathomimetic activity (ISA). This is in contrast to a drug like propranolol that processes no ISA. Theoretically the presence of ISA in β -adrenoceptor blockers suggests that these drugs may be less hazardous in asthma patients. Nevertheless, these drugs should be used with caution in these patients. Other advantages claimed by some investigators include protection against myocardial depression, adverse lipid changes and peripheral vascular complications²¹. The ability of

an agonist to decrease the $R:R^*$ ratio (i.e. increase R^* , thereby "activating" or "stimulating" the receptor), is referred to as its "**efficacy**". The greater the ability to increase R^* , the higher the efficacy of the agonist. In molecular pharmacology, therefore, the term "efficacy" has a different meaning from how it is used in clinical pharmacology, where "efficacy" would refer to how effectively the drug treats the disease or symptoms¹⁷. An agonist is also described as having "**intrinsic activity**", meaning that it is able to give rise to a pharmacological effect. A drug with higher efficacy (receptor activating property) also has a higher intrinsic activity (ability to elicit effect), except when there is a ceiling to the maximal effect in which case higher efficacy does not necessarily increase the intrinsic activity of the agonist in that particular biological system.

- **Inverse agonists:** Some drugs preferentially bind to R over R^* and will drive the equilibrium between R and R^* towards R (decreasing the number of receptors in the R^* state). If there is a significant level of constitutive receptor activity (basal effect), these drugs will decrease receptor signalling and thereby decrease effect. This kind of drug is known as an **inverse agonist** (see *Figure A-3*), giving an effect opposite to that of an agonist¹⁷, i.e. a drug with negative efficacy. If, however, there is not significant constitutive receptor activity, the inverse effect will not be noticeable and the inverse agonists will act as neutral competitive antagonists (see below). Examples of inverse agonists include cimetidine and ranitidine on H_2 -receptors, haloperidol on D_2 -receptors, prazosin on α_1 -adrenergic receptors, timolol on β_2 -adrenergic receptors, clozapine on D_2 - and $5HT_{2C}$ -receptors²² and many experimental drugs such as the benzodiazepine inverse agonist β -CCB and yohimbine on α_{2A} -adrenergic receptors²³. Indeed, many drugs previously believed to be competitive antagonists have recently been shown to act as inverse agonists and it can be expected that several more inverse agonists will be discovered.
- **Neutral competitive antagonists:** Drugs that do not differentiate between R and R^* (i.e. they bind with equal affinity to both conformations) are known as **neutral competitive antagonists** (see *Figure A-3*), i.e. a drug with no efficacy at its receptor. Neutral competitive antagonists do not alter basal receptor activity on their own, but they compete with agonists and inverse agonists for

receptor binding, thereby competitively antagonising the effects elicited by agonists and inverse agonists. A typical example of these drugs is propranolol on β -adrenergic receptors.

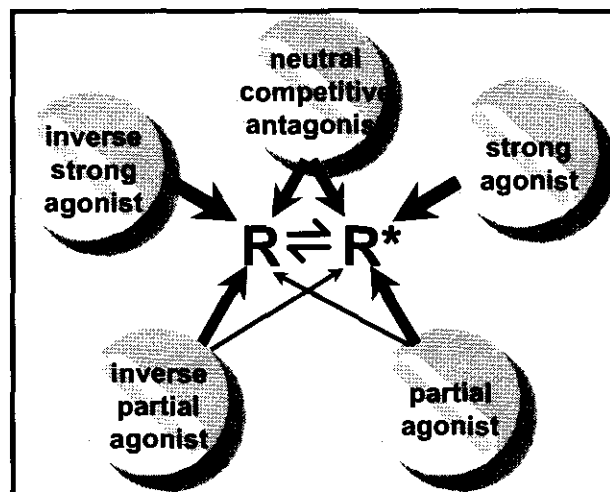


Figure A–3: A schematic representation of how the two-state receptor model relates to the action of drugs as strong agonists, partial agonists, neutral competitive antagonists, inverse agonists, and inverse partial agonists. The inactive and active receptor conformations (R and R* respectively) are in constant equilibrium. A strong agonist binds selectively to R*, driving the equilibrium between R and R* in favour of R*, resulting in enhanced effect. A partial agonist has higher affinity for R* than for R, but with less selectivity than the strong agonist. The neutral competitive antagonist binds with equal affinity to both R and R*, so that it does not disturb the resting equilibrium and therefore does not alter basal effect. An inverse strong agonist binds selectively to R, driving the equilibrium between R and R* in favour of R, resulting in decreased effect, that is, when there is significant constitutive activity (basal effect). An inverse partial agonist has higher affinity for R than for R*, but with less selectivity than the strong inverse agonist.

The clinical significance of the differentiation between neutral competitive antagonists and inverse agonists is not always clear. Much research is currently being done to look into this question. It has been argued that the chronic use of inverse agonists may lead to so called receptor upregulation, as opposed to agonists that usually downregulate receptors, but data on this are not conclusive. To illustrate this principle, the histamine H₂-receptor inverse agonists cimetidine and ranitidine, but not the neutral antagonist burimamide, upregulate H₂-receptors after chronic exposure. The mechanism of receptor up- and down-regulation will be discussed further below under the concept of membrane trafficking of receptors. In addition to the example of H₂ receptors, Kenakin¹⁷ also refers to several examples of disease where constitutive

activity of GPCRs have been observed and where inverse agonists may in future research be proven to have advantages over neutral antagonists, e.g. VIP receptor inverse agonists in the treatment of cancer and also appropriate inverse agonists in the treatment of retinitis pigmentosa, hyperthyroidism, autoimmune disease and certain types of viral infection.

A.2.4 Presynaptic receptors

In the nervous systems we distinguish **pre- and postsynaptic receptors** according to their location. Although postsynaptic receptors have been studied more extensively, a variety of presynaptic receptors have been identified that are of clinical significance. These receptors are important because of their ability to control the release of neurotransmitters^{24,25,26}. Presynaptic receptors facilitate a feedback mechanism whereby they influence (inhibit or promote) the subsequent release of neurotransmitters from the same neuron (autoreceptors) or they may influence the release from neighbouring neurons (heteroreceptors):

- Presynaptic inhibitory **autoreceptors** have been identified on both adrenergic and cholinergic nerve terminals. Activation of these receptors by released noradrenaline or by exogenously administered α_2 -adrenoceptor agonists such as clonidine decreases the further release of the neurotransmitter. The same subtype of α_2 -adrenoceptors inhibits the release of acetylcholine from cholinergic neurons²⁷. Other inhibitory autoreceptors are also described, for example for dopamine (D_2/D_3 -receptors), acetylcholine (M_2 -receptors), GABA ($GABA_B$ -receptors), histamine (H_3 -receptors) and serotonin ($5HT_{1D}$ -receptors)²⁴. In addition to the presynaptic inhibitory autoreceptors, there are also presynaptic autoreceptors that enhance the release of the neurotransmitter, including acetylcholine (nicotinic) and noradrenaline (β_2) presynaptic receptors.
- Presynaptic **heteroreceptors** are receptors that modulate neurotransmitter release when they are stimulated by neurotransmitters other than the neurone's own transmitter that are present in the synaptic cleft. For example, noradrenaline nerve terminals possess presynaptic facilitatory angiotensin II receptors and presynaptic inhibitory opiate receptors^{28,29,30}.

Presynaptic receptors are suitable targets for exogenous drugs such as agonists or antagonists. Consequently these receptors are targets of action for a new generation of drugs that may intervene selectively at the level of presynaptic release-modulating receptors²⁴. Examples of the latter are the antidepressant mirtazapine that antagonises α_2 -adrenoceptors and modulates the release of noradrenaline and serotonin^{31,32}, and the neuroleptic amisulpride that is a selective antagonist at D₂/D₃-dopamine autoreceptors that modulate the release of dopamine²⁴. The improved efficacy of the atypical antipsychotics, such as clozapine and olanzapine, in the treatment of schizophrenia, and their lower incidence of motor side-effects, has been linked to their ability to promote dopamine release *via* actions at auto- and heteroreceptors in the limbic and striatal regions of the brain².

A.2.5 G proteins and signalling

As discussed above, transmembrane GPCRs may activate G proteins on the inner surface of the cell membrane to continue the signal transduction initiated by the drug binding to the GPCR¹¹. The G proteins are composed of three subunits, namely the α -, β and γ subunit (hence the heterotrimeric character of G proteins), where the β - and γ -subunits function as a unit (see *Figure A-1*). The G proteins can be subdivided into four families (namely G_s, G_{i/o}, G_{q/11} and G_{12/13} proteins)³³ where GPCRs show selectivity for coupling to these respective G protein families. It has been found that G_s proteins primarily activate adenylyl cyclase, G_i proteins primarily inhibit adenylyl cyclase, G_q proteins primarily activate phospholipase C and G_{12/13} proteins primarily regulate small GTP binding proteins (not mentioning, of course, other G protein effectors). Within these G protein families, researchers have identified several different subtypes of α -, β and γ -subunits and combinations thereof to even further complicate this classification, although there are still many investigations and discussions about their relevance and function^{12,34,35,36}. The same subtypes of subunits may also be further subdivided into splice variants that may be expressed differently in different tissue or with age³⁷.

To understand how potential future drugs, such as the RGS-modulating drugs discussed below, may modulate G protein function (and consequently signal transduction), it is important to understand more about the mechanism by which G

proteins function. In the inactive state, the α -subunit of the G protein is bound to GDP. The complex formation between the active receptor state and the G protein is followed by the release of GDP from the α -subunit of the G protein, subsequently allowing for a GTP molecule to bind. The G protein is now activated and the α - and $\beta\gamma$ -subunits become separated (see *Figure A-1*). The active GTP-bound α -subunit is sometimes referred to as $G\alpha_{(GTP)}$ and the $\beta\gamma$ -subunit as $G\beta\gamma$. These presumably mobile subunits are known to influence cell function by, for example, altering enzyme function (e.g. adenylyl cyclase or phospholipase C) and the consequent production of second messengers (e.g. cAMP or inositol-1,4,5-triphosphate (IP_3) and diacylglycerol) or to alter ion channel function³⁶.

Within a fraction of a second the GTP on the $G\alpha_{(GTP)}$ is hydrolysed to GDP by GTPase on the $G\alpha$ and this allows for the α - and $\beta\gamma$ -subunits to re-unite and to form inactive G protein complexes again.

A.2.5.1 RGS modulating drugs

Regulators of G protein Signalling (RGSs) are a family of proteins that can modify (regulate) the signal transduction by G proteins. Because of their important physiological function combined with several other special properties, they are important potential drug targets. Their proposed clinical significance will be discussed further below. RGSs regulate G protein signalling in any of the following ways^{36,38,39}:

- **GAP function:** As mentioned earlier, the α -subunits of G proteins have inherent GTPase activity that hydrolyses GTP to GDP, thereby self-inactivating $G\alpha_{(GTP)}$ back to $G\alpha_{(GDP)}$. The GTPase activity is inherently too low for normal physiological functioning, but can be regulated (greatly enhanced) by the RGSs, so that they accelerate the inactivation of $G\alpha_{(GTP)}$ to $G\alpha_{(GDP)}$ and thereby accelerate the G protein cycle. As a result RGSs will also shorten any R^* and agonist- R^* mediated signalling duration and thereby weaken agonist action, as is illustrated in *Figure A-1*. Since this function of the RGSs relates to enhanced GTPase activity, RGSs with this function are also referred to as **GTPase activating proteins (GAPs)**. The GTPase activating

function of RGSs is therefore sometimes referred to as their GAP function. It has been found that the majority of RGSs function as GAPs.

- **Non-GAP function:** However, not all RGSs function as GAPs. RGSs may also directly antagonise G α effectors, thereby preventing G α from signalling to its effector. Other functions and mechanisms (of lesser importance for the purpose of this review) have also been proposed. For example, RGSs may antagonise G $\beta\gamma$ -subunits by binding to e.g. the β -subunit, thereby preventing the unit from performing its physiological role. RGSs may also directly bind to receptors or act as G α effectors to modulate signal transduction pathways.

RGSs are now regarded as important drug targets. Currently there are no drugs registered as RGS modulators, but active research is being conducted and the following arguments support RGS modulating agents as potential drugs of the future:

- **Several families** of RGSs (each with subfamily members) have been identified so far. RGS subtypes may show selectivity for various G protein subtypes, so that it may be possible to target specific G proteins by targeting specific RGSs with modulating drugs³⁸.
- Importantly these various types of RGSs have been suggested to be **expressed selectively and differentially in various tissues**. RGS7 is expressed at higher levels in the neocortex, hippocampus and certain nuclei than in other brain regions³⁸. RGS4 is abundant in many brain tissues, but its expression can be regulated differently in different brain regions following stress or corticosterone administration (specifically a decrease in paraventricular nucleus and pituitary and increase in locus coeruleus)³⁸.
- **Altered RGS expression in disease** has been reported. In schizophrenia, for example, RGS4 expression is decreased in the prefrontal cortex (as determined by microarray analysis of mRNA in post-mortem brain). It is believed that this may account for typical symptoms of schizophrenics in situations of stress⁴⁰. Moreover, in Parkinson's disease it has been shown that the levels of RGS9 are increased in both the caudate and putamen, relating Parkinson's disease with decreased D₂-receptor signalling⁴¹. It has also been proposed that RGS proteins associated with G_q and G_i proteins may be linked to embryonic

cardiomyocyte proliferation, as well as cardiac hypertrophy and cardiovascular adaptation to pressure overload and physiological stress in adults³³.

Any selective inhibitor of a particular RGS type will enhance agonist activity, making this an attractive approach for drug discovery. The above-mentioned data on Parkinson's disease and RGS9 suggests that a RGS9 antagonist may be of therapeutic benefit in patients with Parkinson's disease. Likewise a RGS4 agonist (or potentiating agent) may be of therapeutic benefit in schizophrenic patients. This therapeutic potential underscores why current research is focusing on the development of selective RGS modulating agents as novel drugs of the future³⁹. Whether this strategy will enable us to modify GPCR-mediated signalling without affecting associated problems such as receptor sensitisation or desensitisation (see membrane trafficking below), however, still needs to be addressed.

A.2.5.2 Fine-tuning GPCR signalling

Although the human body expresses probably thousands of different types and subtypes of GPCRs, the number of GPCR effector types (e.g. second messenger systems) is far less¹². It is therefore obvious that the body needs extraordinary and delicate mechanisms to differentially regulate the various diverse cellular functions with only a limited number of signalling pathways⁴². Researchers have put forward explanations such as the **specificity of GPCR coupling with G proteins**^{9,11,34}, **membrane organisation**^{9,35,43,44,45} and signalling **cross-talk mechanisms**^{12,46} to explain this phenomenon.

Cross-talk suggests that signalling pathways may be interlinked (can merge at some point), so that one type of GPCR may influence the signalling of another (for example the SSRI-induced 5HT-receptor-dopamine cross-talk described in the introduction of this article). Cross-talk is believed to be a general phenomenon, since most cell systems have a large number of different GPCR types with a much more limited number of effector (e.g. second messenger) systems¹². A particular GPCR type may couple to more than one G protein type (receptor heterogeneity/promiscuity) or, conversely, different GPCR types may couple to the same metabolic pathway of even the same G protein type^{16,46}. A particular receptor (R_x) may therefore be able to couple to both G protein types G_1 and G_2 in a cell, or conversely, if both receptor 1 (R_1) and

receptor 2 (R_2) are able to couple to a particular G protein (G_x), it may be possible that both R_1 and R_2 activate G_x in the cell (see *Figure A-4*). To illustrate this phenomenon, α_{2A} -adrenoceptors can couple to G_i , G_s and G_q proteins (although with different strength)^{44,47,48} and conversely both serotonergic 5HT_{2A}-receptors and muscarinic acetylcholine M₅-receptors are known to couple to G_q proteins^{27,49}. Thereby one cellular effect may result from a fine balance of several substances, and not merely by the fluctuation of the concentration of one substance. In the clinical setting, cross-talk is not a new concept. It has been used to explain, for example, why antidepressants of different neurotransmitter selectivity (e.g. *l*-noradrenalin or serotonin) ultimately evoke the same neuronal effect regardless of receptor selectivity. Examples of this form of unpredictable post-receptor cross-talk include similar antidepressant-induced changes in biogenic amine metabolites and down regulation of β -adrenoceptors in limbic regions of the brain that correlate with improvement in depressive symptoms^{2,50}. The implications are that these far-reaching inter-regulatory elements allow a particular underactive/overactive pathway to be modulated, despite the drug's having a "select" action on another extracellular receptor system. *Figure A-5* illustrates various cell-surface receptor-linked pathways utilizing G protein-coupled second messenger systems and how these pathways may interact at the post-receptor level, e.g. at second messenger level (e.g. cAMP-phospholipase C), at enzyme level (e.g. cGMP-phosphodiesterase), at protein kinase level and at the G protein level (e.g. G_s - G_q interactions). Activation, or inhibition, of one particular extracellular receptor may modulate events set in motion by separate extracellular receptor effects².

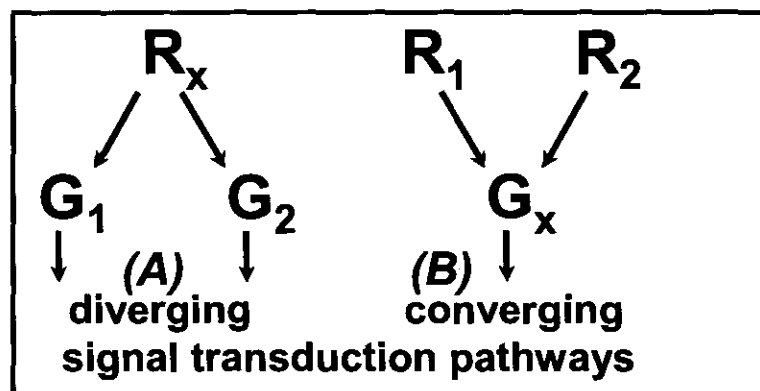


Figure A-4: A schematic representation of how receptor promiscuity may lead to either the divergence of one signal transduction pathway into several downstream pathways or the convergence of signal transduction pathways into one pathway. **(A)** R_x represents a single GPCR type that couples to two different G protein types G_1 and G_2 , thereby diverging the signal into two independent signal transduction pathways. **(B)** R_1 and R_2 are two different GPCR types that both couple to a particular G protein type G_x , so that their signals converge into one signal transduction pathway.

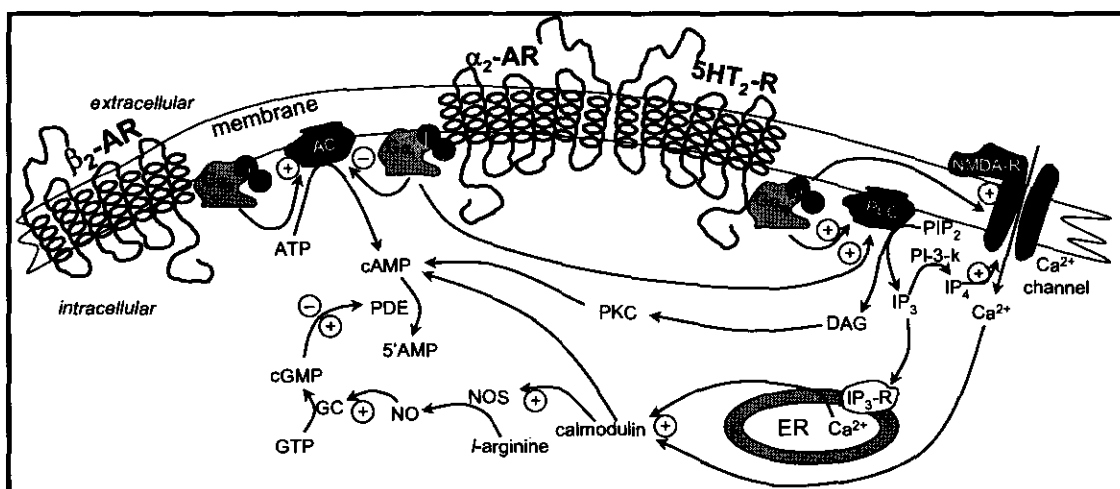


Figure A-5: A schematic representation of receptor cross-talk, illustrating various examples of GPCR signal transduction pathways, where β_2 -AR = beta-2-adrenergic receptor; α_2 -AR = alpha-2-adrenergic receptor; $5HT_2$ -R = serotonin type 2 receptor; NMDA-R = N-methyl-D-aspartate receptor; ER = endoplasmic reticulum; AC = adenylyl cyclase; PLC = phospholipase C β ; PDE = phosphodiesterase; PKC = protein kinase C; ATP/GTP = adenosine/guanosine triphosphate; cAMP/cGMP = cyclic adenosine/guanosine monophosphate; PIP₂ = phosphatidyl inositol biphosphate; IP₃/IP₄ = inositol tri/tetra-phosphate; NO = nitric oxide; NOS = nitric oxide synthase; ⊕ = stimulating effect; ⊖ = inhibitory effect.

Another example of where future therapy may benefit from our understanding of cross-talk, is in the treatment of Parkinsonism. This severely debilitating disease is caused by the progressive degeneration of dopaminergic neurotransmission from the

mesencephalon to the striatum. Current therapy involves the replacement of central dopamine, but is often associated with a progressive decrease in efficacy and increase in dyskinesias. Recent research indicates cross-talk between serotonergic 5HT_{1B}, dopaminergic D₂- and cannabinoid CB₁-receptors. Since these receptors are shown to be co-localised, it was suggested that their signal transduction systems may converge⁵¹. In this regard, it has also been shown that D₁-receptor-mediated activation of adenylyl cyclase can be completely blocked by CB₁ stimulation and, conversely, that dopamine receptors regulate the release of endocannabinoids. In addition, it has been suggested that in Parkinsonism, the brain might normalise striatal function by elevating striatal endocannabinoids and CB₁-receptors (receptor up-regulation). This has led to the proposal that new cannabinoid-based drugs and inhibitors that reduce the enzymatic breakdown of these derivatives might be useful in treating Parkinsonism⁵². One such plant alkaloid, a main psychoactive component of *Cannabis sativa* (dagga), exerts its effects by interacting with cannabinoid receptors⁵¹.

Membrane organisation, on the other hand, would suggest that different GPCRs and G proteins are concentrated in specialised and distinct microdomains on the cell membrane, as opposed to random distribution or freely diffusible systems^{12,35}. For example, if R₁ and R₂ are able to couple to a G_x, it may be possible that only R₁ activates G_x in the cell, simply because R₁ and G_x are co-localised, whereas R₂ is located in a different microdomain. In a second cell type, R₂ and G_x may be co-localised and therefore R₂ can activate G_x. Membrane organisation would therefore provide us with an explanation for differences in the regulation of the same signal transduction pathway in different cells and also explains why the signals of two receptors linked to the same G protein do not necessarily merge. The significance of the close association of signalling proteins in cellular microdomains is not yet fully understood, but it may serve to recruit important components, thereby enhancing efficiency and rapidity of coupling, or to hold signalling molecules inactive until needed or even to attract signalling components to terminate the signal¹².

Agonist-directed trafficking of receptor signalling (ADTRS) is a recent concept in molecular pharmacology that also may impact on clinical therapeutics in the future^{17,53,54,55}. GPCRs are known for their heterogeneous coupling to G proteins, meaning that a particular GPCR type may be able to couple to more than one type of G

protein. As an example, α_{2A} -adrenergic receptors are able to couple to G_i , G_s and G_q proteins^{44,47,48}, but with different affinity. The following cases can be distinguished:

- In cells containing all three of these G proteins, it is predicted by **classical theory** that a maximal concentration of a strong agonist will be able to cause the activation of all three G protein types to the maximum (100%), while a maximal concentration of a partial agonist will cause equal but partial activation of all three G proteins (i.e. <100%). Therefore, if a particular agonist is able to cause activation of one type of G protein type (e.g. G_i proteins) to a certain percentage of its maximum, it will also cause the activation of the other types of G proteins (e.g. G_q and G_s proteins) to exactly same percentage of their maximum⁵³.
- According to the concept of **ADTRS**, however, there may be agonists that activate the GPCR in such a way that it selectively activates only one G protein type. An example of such action is to be found in the weak α -adrenoceptor agonist, *l*-isoproterenol, that activates α_2 -adrenergic receptors in such a way that it selectively activates G_s , as opposed to G_i proteins⁵⁴. Similar findings were reported for serotonergic 5HT₂-receptors⁵³. This can be explained by assuming a model where different active receptor states (R^* and R^{**}) are responsible for activating the different G proteins (see *Figure A-6*). Agonists that selectively promote only one active receptor state (i.e. R^* or R^{**}), have been referred to as **ligand-selective agonists** or receptor active-state selective agonists. Since different G proteins activate different metabolic pathways (or activate the same pathway differently), ADTRS allows selective activation of only one metabolic pathway by a receptor. If, for example, one metabolic pathway accounts for unwanted side-effects and the other for the beneficial therapeutic pathway of most agonists of a receptor type, then an agonist that selectively activates the therapeutic pathway will be beneficial. In the search for new drugs, it is therefore important not only to search for drugs that are more selective for particular receptor subtypes, but also for drugs that may work on the same receptor, but preferentially activates appropriate metabolic pathways or that selectively inhibits the inappropriate metabolic

pathway. As a relatively novel concept, ADTRS has not found place in the clinical therapeutic setting as yet.

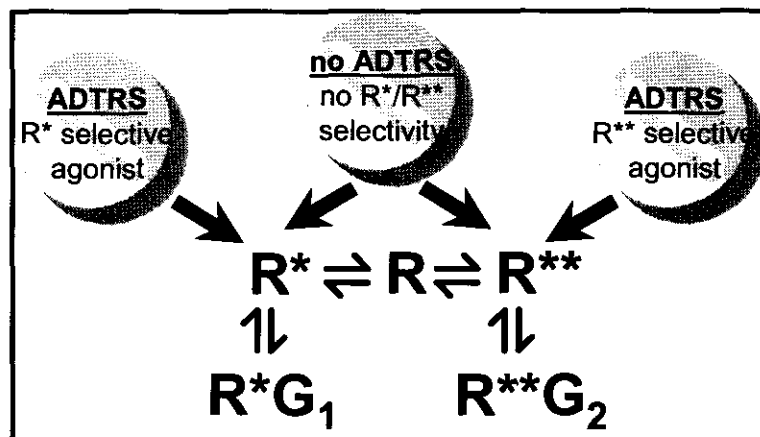


Figure A-6: A schematic representation of how the three-state receptor model for GPCRs explains the phenomenon of agonist-directed trafficking of receptor signalling (ADTRS). R is the inactive receptor state, R* the active receptor state coupling to and activating G protein type 1 (G₁) and R** is a second active receptor state coupling to and activating G protein type 2 (G₂). R, R* and R** are in constant equilibrium. Agonists that binds equally well to R* and R** will not display ADTRS, whereas agonists with selective binding to either R* or R** will favour coupling of the GPCR to either G₁ or G₂ respectively, thereby selectively activating one signal transduction pathway and therefore displaying ADTRS.

Protean agonism is another interesting concept in molecular pharmacology that is related to ADTRS. This unique type of agonism describes a situation where a particular agonist is able to present itself as an agonist in one system and as an inverse agonist in another system, but at the same receptor^{17,56,57}. Protean agonism is currently being researched and there are several examples of drugs that have been suggested to act *via* this mechanism. The mechanism behind protean agonism is rather complex and falls beyond the scope of the review. As with ADTRS, protean agonists may, at least in theory, hold potential therapeutic benefits in that it may reveal agonism selective to one tissue, while acting as inverse agonist or antagonist in another.

Receptor function may further be regulated by **receptor trafficking**, whereby the cell regulates the number of available receptors in the membrane. The body uses this mechanism to prevent continuous over stimulation of a particular receptor. GPCR trafficking is also believed to play an important role in drug abuse, for example with the opioids (morphine and related drugs) and hallucinogens (e.g. lysergic acid diethylamide

or LSD), where it causes tolerance associated with typical drug seeking behaviour⁵⁸. The chronic use of antidepressants (including several tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors and atypical antidepressants) and antipsychotics have also been associated with the down-regulation of serotonergic 5HT_{2A}-receptors. Paradoxically, both agonists and antagonists at 5HT_{2A}-receptors have been shown to down regulate these receptors⁵⁹. It has also been suggested that future drugs that regulate the trafficking of dopaminergic receptors may have therapeutic value in Parkinson's disease⁶⁰.

Receptor trafficking typically includes the processes described below^{14,59,61} (for an animation of agonist-induced activation of GPCRs, receptor desensitisation, internalisation and eventual recycling, see:

<http://archive.bmn.com/supp/tips/tips2210a.html>)¹⁴:

- 1. Receptor desensitisation:** Receptor desensitisation is the initial process whereby an agonist-bound receptor is phosphorylated by G protein-coupled receptor kinases (GRKs). The phosphorylated receptor subsequently binds to so called arrestins to potentiate the desensitisation, rendering the receptor non-functional. This is a rapid process that may occur within seconds after agonist stimulation and can be viewed as a negative feedback mechanism whereby the body prevents the excessive stimulation of a particular receptor. The process whereby an agonist desensitises its own receptor is known as **homologous** receptor desensitisation, such as is found with the overuse of β_2 -adrenoceptor agonists (bronchodilators such as salbutamol) in asthma. It is, however, also possible that stimulation of one receptor may desensitise another, known as **heterologous** receptor desensitisation, where generally protein kinase A or C (and not GRK) is responsible for the receptor phosphorylation.
- 2. Receptor sequestration and internalisation:** Once desensitised, GPCRs are scaffolded to specific membrane regions where the membrane folds inward (invagination) to form vesicles (so-called clathrin-coated pits), enclosing the GPCRs. These GPCR containing vesicles are released into the cellular cytoplasm by a process called internalisation. Some GPCRs (e.g. muscarinic

acetylcholine receptors) are internalised *via* caveolae (a smooth non-clathrin vesicle, containing caveolin), but this alternative endocytosis pathway for GPCRs needs more thorough investigation.

- 3. Receptor degradation or recycling:** Once internalised, the GPCR can be either metabolised by lysosomes (**down-regulation**) or dephosphorylated and recycled to the cell membrane to restore function (**resensitisation**).

Research data suggest that desensitisation and down-regulation can be dissociated, implying that a drug may, for example, cause desensitisation without down-regulation. Also, since it is assumed that receptor internalisation may be necessary for dephosphorylation and recycling (resensitisation) of desensitised receptors, drugs that inhibit receptor internalisation but not receptor desensitisation may actually lead to an increased number of receptors remaining in the desensitised state⁵⁹. Studies suggest that an increase in the number of desensitised μ -opioid receptors may be associated with treatment with morphine⁶² and fentanyl⁶³. This increase in the number of desensitised receptors is presumably responsible for the tolerance and associated drug seeking behaviour. Although both morphine and etorphine induce tolerance after seven days treatment in mice, only etorphine produces μ -opioid receptor downregulation⁶⁴. Similar results have been obtained in cell cultures⁶⁵. These results suggest that opioid agonists may regulate trafficking proteins differentially. It has also been shown that L-type calcium channels may be involved in μ -opioid receptor trafficking, since calcium channel blockers, such as nimodipine, are able to prevent μ -opioid receptor down-regulation by agonists⁶⁶. In addition, it has been shown that the body controls chronic inflammatory pain by increasing δ -opioid receptor expression (receptor up-regulation) and the recruitment of intracellular receptors to the plasma membrane, thereby decreasing pain. This finding has initiated the challenge to develop endogenous enkephalin-like peptides for controlling inflammatory pain⁶⁷. All of the above allows us to appreciate not only the complexity of this phenomenon, but also illustrates the importance thereof for drug interactions in therapeutics.

Dimerisation and oligomerisation of GPCRs is another mechanism whereby the fine-tuning of the signal transduction systems is accomplished. Dimerisation implies structural complex formation between two GPCRs to operate/function together, whereas oligomerisation would imply more than two GPCRs in such a complex. We

also distinguish between homodimerisation and heterodimerisation, where the former would imply that two identical GPCRs would form a complex, whereas the latter imply that two different types of GPCRs form a complex. Dimerisation may be necessary for efficient agonist binding and signalling or may even generate new drug binding sites^{8,14}. It has been shown that adenosine A₁- and dopaminergic D₁-receptors form dimers, suggesting that future drug treatments for Parkinson's disease may be targeted at adenosine receptors rather than at dopaminergic receptors⁸.

Different **isoforms of GPCRs** (differences in amino acid sequence) have been implicated in specific disease states, resulting from alternative splicing or mRNA editing. These substitutions of one or more amino acids within the GPCR protein may lead to altered activity or ligand-binding properties of the receptor. Pre-mRNA editing has been shown to exist for the serotonergic 5HT_{2C}-receptor in the human prefrontal cortex of depressed suicide victims. Strikingly, fluoxetine has also been shown to cause mRNA editing in mice exactly opposite to that seen in the depressed suicide victims, suggesting that it may reverse such abnormalities in humans⁶⁸. Interestingly, G protein mutations, with resulting signalling altered function, have been observed in hypertensive patients, patients with testotoxicosis and patients with type I pseudohypoparathyroidism⁶⁹.

A.3 Conclusions

Understanding molecular receptor and signal transduction pharmacology enables practitioners to improve their understanding and effective implementation of current and future pharmacotherapy. It helps practitioners to understand and predict possible drug interactions, develop and improve therapeutic strategies and with subsequent enhancement of the quality of life of their patients. According to Kenakin¹⁷ the challenge for the next millennium in drug discovery and receptor pharmacology will be to exploit the complex pharmacological properties of the drugs acting on GPCRs for therapeutic advantage. New findings in the near future in the field of GPCRs will indeed lead to novel therapeutic approaches aiming at the optimisation of drug therapies.

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Instructions to the Authors:
British Journal of
Pharmacology
(Appendix to Chapter 3)

Appendix
B

A copy of the "Instructions to the Authors" of the *British Journal of Pharmacology* follows on the next page.

INSTRUCTIONS TO AUTHORS

With effect from February 2003

Manuscripts can be submitted using the following options:

- Log onto the BJP Website www.bjppharmacology.org and submit to e-Journal Press (BJP online submission web page).
- Mail one hard copy accompanied by a disk to the BJP Editorial Office.
- British Journal of Pharmacology Editorial Office
- 16 Angel Gate, 326 City Road, London EC1V 2SG
- Tel: +44 (0) 207 417 0432 Fax: +44 (0) 207 417 0430

FULL PAPERS

Manuscripts must be typed in double-line spacing, in type not smaller than 11 point with margins of no less than 3 cm and each page should be numbered. If submitting a hard copy manuscript should be printed on one side of A4 paper and clipped (not stapled) together. Papers in recent issues of the *British Journal of Pharmacology* should be consulted for the general layout of the paper and also for details. For all manuscripts the following subsections are used:

- Title page
 - Summary
 - Introduction
 - Methods
 - Results
 - Discussion and conclusions
 - Acknowledgements
 - List of references
 - Tables
 - Legends
 - Figures
- Preferred file formats are: Word for Windows, Wordperfect for Windows, Microsoft Word for Mac, or ASCII. For non electronic submission please supply the manuscript (including all Tables and Figures) plus a copy on disk (please label your disk with the corresponding author's name, file name and programme used).

Title page
The title should normally contain no more than 150 characters and should not be interrogative or consist of more than one sentence. It should clearly indicate the subject matter of the paper and any statements contained therein should be justified by the results presented in the paper. A short running title containing not more than 50 characters and spaces is also required. The title page should include the names of authors and their appropriate addresses. It should be made clear which address relates to which author. Authors' present addresses differing from those at which the work was carried out should be given as footnotes on the title page and references at the appropriate place in the author list by superscript numbers. A footnote may also be used to indicate the author to whom correspondence should be sent. Please note an e-mail address must be given. The use of footnotes for any other reason is not allowed. If the e-mail address to which proofs should be sent is not that of the first mentioned author, clear instructions should be given in a covering note and not on the title page. The title page should be paginated as page 1 of the paper.

The *British Journal of Pharmacology* welcomes contributions in all fields of experimental pharmacology for publication as full papers or as high priority Special Reports. Review Articles and Commentaries. (Please note Commentaries must be commissioned by the Journal.)
Papers should normally be based on new results obtained experimentally and should constitute a significant contribution to pharmacological knowledge. Papers which reassess pharmacological concepts based on earlier results will also be considered as will purely theoretical papers. Papers dealing only with descriptions of methods are acceptable if new principles are involved.
Contributions that have already been published, or accepted for publication, must be signed as a hard copy by all authors and returned to the BJP editorial office. All signatures must be hard copy originals – faxed or scanned figures will not suffice. This licence concerns the originality of the submitted paper and does not constitute an endorsement of the information available in the previously published contribution.
Papers are only accepted if accompanied by a licence to publish which must be signed as a hard copy by all authors and returned to the BJP editorial office. See page 721 for details.
The Journal will not consider papers which describe experiments on animals which do not fall clearly within the current laws governing animal experimentation in the United Kingdom (www.archive.official-documents.co.uk/document/hoc/321/321-00.htm). Authors must make it clear that the procedures they used were as humane as possible and complied with the guidelines for animal care of their institutions or with national/international guidelines. The doses (initial and subsequent) of anaesthetics and analgesics should be clearly stated; the method of assessing anaesthesia, particularly after the administration of skeletal muscle relaxants (neuromuscular blocking drugs), must be well defined. The Society has an Ethics Committee which can be consulted by authors through the Senior Editor to the Editorial Board.

When investigations on normal human subjects are reported, evidence of approval by a local Ethics Committee must be given. Papers concerned with clinical trials on the effects of drugs on patients are not appropriate for this Journal.
Authors are strongly urged to keep their manuscripts as short as they reasonably can. An effective way is to reduce the Discussion and the number of figures to a minimum and to avoid repetition of information that has already been published. Authors should remember that a reader may be influenced by literary style and will appreciate simple but accurate prose. The Journal provides a language Editing Service for its authors. For further information on this service please contact eam@bps.ac.uk.
It is important to note that failure to comply with these Instructions to Authors may lead to editorial delays. Authors may wish to suggest the Senior Editor they consider most suitable, along with the names of two possible Editors to review their manuscript. The final selection will, however, remain with the Senior Editor.
Editors to review their manuscript. The final selection will, however, remain with the Senior Editor.
Instructions to Authors may lead to editorial delays. Authors may wish to suggest the Senior Editor they consider most suitable, along with the names of two possible Editors to review their manuscript. The final selection will, however, remain with the Senior Editor.

Summary

The summary will be printed at the beginning of the paper. It must not exceed 250 words and should be intelligible to the non-specialist reader and suitable for direct transposition by abstracting services. It should contain a brief account of the question addressed in the paper, the principal methods and results, and the main conclusion(s), and should be arranged in numbered and concise paragraphs. Abbreviations and symbols should be explained in brackets on first use – for example, bradykinin (BK). References should be avoided where possible; if considered absolutely necessary, they should include the first or only two authors, year, journal abbreviation and volume and page numbers, for example (Fenwick *et al.*, 1982; *J. Physiol.*, 331, 599–635; Takahashi & Momiyama, 1993; *Nature*, 366, 156–158). **Keywords.** Up to 10 keywords or phrases of two to three words (including names and terms used in the title) should be displayed at the end of the summary. Keywords will be used to compile the annual index. The quality of the index will thus be determined by the appropriateness of the keywords. These may be selected by reference to the most recent Index of the Journal. Avoid unhelpful or unqualified terms such as 'inhibition', 'drug' etc. **Abbreviations.** An alphabetical list of non-standard abbreviations should be provided at this point – for example HUVEC, human umbilical vein endothelial cells; VSMC, vascular smooth muscle cells. (The full name plus abbreviation should also be used in the text on first mention.)

Introduction

The introduction should give a short and clear account of the background of the problem and the rationale of the investigation. Only previous work that has a direct bearing on the present problem should be cited.

Methods

The methods must be described in sufficient detail to allow the experiments to be interpreted and repeated by the reader. However, detailed repetition of methods which have been adequately described previously should be avoided and references given, although a brief outline is often helpful.

The methods are presented in sub-sections, usually defined by the experimental procedure. Where animals are used, they and their conditions of maintenance (food, water, light/dark cycles and ethical guidelines) should be described in the first subsection of Methods. The penultimate subsection should list all drugs, chemicals and other consumable materials (cells, culture reagents and solutions, antibodies, assay kits, etc) with the names and brief addresses of their suppliers.

Drugs, chemicals and other materials (cell culture reagents, antibodies, etc) should be listed in a separate paragraph after the methods. This paragraph should also include the names and brief address of the relevant suppliers. Drug names should be 'approved names' as published previously in the British Approved Names 2001 (www.tso.co.uk/bookshop/bookstore.asp?ACTION=Book&productID=011322558X) or as listed in the current version of the British National Formulary (www.bnf.org/webnf/form1/bnf/index.html). If a drug has no 'approved name' its chemical name must be used and the rules set out in the current *Handbook for Chemical Society Authors* (London, Chemical Society – www.rsc.org) observed, or its structural formula given. Cumbersome chemical names should be suitably abbreviated for later reference in the paper.

The doses of drugs should be given as unit per body weight, e.g. mmol kg⁻¹ or mg kg⁻¹; concentrations should be given in terms of molarity, e.g. nM or μ M.

The last subsection should provide the methods of data analysis and statistical assessment that have been used.

Results

The description of the experimental results should be succinct but, nevertheless, in sufficient detail to allow the experiments to be repeated by others. Typical single experiments may be presented with a clear statement that *n* number of similar experiments had similar results. Where appropriate, however, the mean results with confidence limits or with standard errors of the means and the number of observations should be given. Statistical tests of significance should be performed where appropriate. The results of such tests should be stated as the numerical value of the probability (*P*) that is calculated, with any necessary clarification (e.g. one-tail or two-tail test).

Every effort should be made to avoid unnecessary repetition of data in the text, tables and figures. Conclusions and theoretical considerations should not be elaborated in this section.

Discussion

The purpose of the discussion is to present a brief and pertinent interpretation of the results against the background of existing knowledge. Any assumptions on which conclusions are based must be stated clearly. A mere recapitulation of the results is not acceptable. A review-like treatment, which reduces the impact on the reader, should also be avoided. The main conclusion should be conveyed in a final paragraph.

Acknowledgements

Acknowledgements should be brief but should include reference to sources of support. Sources of drugs not widely available commercially should be acknowledged.

References

In the text, references to other work should take the form: (Bolton & Kitamura, 1983) or, 'Bolton & Kitamura (1983) showed that...'. If there are more than two authors, the first author's name should be given followed by *et al.* (Bülbring *et al.*, 1981).

References to 'unpublished observations' or 'personal communications' should be mentioned in the text only, and not included in the list of references. Papers which have been submitted and accepted for publication, should be included in the list of references with the names of the periodicals and 'in press'. The Editor reserves the right to request a copy of any papers in press. Papers in preparation or which have been submitted but not yet finally accepted for publication must not be included in the list of references.

The reference list at the end of the manuscript must be arranged alphabetically according to the surname of the first author. When the surnames of first authors are identical, the alphabetical order of the surnames of subsequent authors takes precedence over the year of publication. The AUTHORS' names are followed by the year of publication in brackets. If more than one paper by the same authors in one year are cited, a, b, c, etc. are placed after the year of publication, both in the text and in the list of references. The title of the article is given in full, followed by the abbreviated title of the periodical, volume number and first and last page numbers. The abbreviations used for periodicals are those of the most recent edition of the International List of Periodical Title Word Abbreviations. References to articles in books should consist of names of authors, year of publication, title of article followed by the title of the book, the editors, volume number, if any, and page numbers, the

place of publication and the names of the publishers. For example:

BOLTON, T.B. & KITAMURA, K. (1983). Evidence that ionic channels associated with the muscarinic receptor of smooth muscle may admit calcium. *Br. J. Pharmacol.*, **78**, 405–416.
BRADING, A.F. (1981). Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In *Smooth Muscle: An Assessment of Current Knowledge*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

Tables

Each table should be given on a separate page, paginated as part of the paper. Tables should be numbered consecutively with arabic numerals and the number should be followed by a brief descriptive caption, occupying not more than two lines, at the head of the table. The proportions of the text area should be borne in mind when designing the layout of tables. For the sake of clarity, tables should not have more than 120 characters to a line, with spaces between columns counted as four characters. The absolute maximum is 180 characters to a line. Each column should have a heading and the units of measurement should be given in parentheses in the heading. Except in special circumstances, tables should be self-explanatory; the necessary descriptions should be at the bottom of the table.

Figures

To avoid unnecessary Figures, particularly those requiring half-tone reproduction, only critical points of the text should be illustrated. The cost of colour Figures will be charged to the Author. Upon acceptance, Authors will be notified of their colour charges by the Production Office. Please note that unsatisfactory Figures will be returned to the Author for revision. The Journal reserves the right to reject a manuscript if the Figures are unacceptable.

Submission Requirements

- Preferred size for illustrations is 80 mm single column or up to 160 mm double column. All illustrations will be reduced to fit single column width wherever possible.
- If the artwork has been created to the correct size the labelling placed around the illustration should be 8 pt Univers. Otherwise, labelling should be set to the correct percentage for reduction at page make-up. Amendments to the illustrations (i.e., labelling) may be made to conform to the journal style.
- Figure legends should be typed on a separate page. Legends should explain the Figures in sufficient detail that, whenever possible, they can be understood without reference to the text. Figure legends/captions should be consistent with terminology or nomenclature used in the labelling of the Figures.
- The explanation of symbols must be given as a key in the Figure itself and not in the Figure legend.
- Please provide one full set of labelled Figures (i.e. complete with lettering and numbering, arrows, etc.). Please label the file with the corresponding author's name. Please state if figures have been submitted separately and if so also label figure files with author's name.
- Any illustrations that have been prepared on a computer may be submitted in electronic form. Preferred file formats are TIFF or JPEG. Any colour figures should be saved as CMYK, not RGB.

- Minimum resolution to be 300 dpi for colour Figures or black and white halftones and 600 dpi for line illustrations.
- Any illustrations containing blots from gels, histochemical stains or pen traces, that have been prepared via a computer program cannot be reproduced from a laser printout, as this creates a cross-hatched pattern. Such material must be submitted on disk, unless unscanned continuous tone originals are supplied.

Line Figures

If not submitted electronically (as above) line illustrations should be presented as clear black and white artwork (camera ready copy), with suitable contrast enabling them to be scanned directly into a printable format. They should be prepared to conform with the style and convention of the journal as redrawing is expensive and processing time is extended.

Journal style for lettering on figures is plain sans serif typeface (Univers). Most figures will be reduced in size for reproduction, final type size is generally 8pt (after reduction). Artwork may be submitted up to twice the intended size in the journal (see Figure 1).

Subsection figure parts (a, b, etc.) should be labelled in bold lower case (8pt, Univers bold) to match figure labelling.

It is important that the printed symbols and lines should retain their clarity. To achieve this the following points should be considered:

1. Lines should not be too thin to reproduce after reduction to on-page size (see Figure 1).
2. The symbols used for plotting data points should be large enough to show up clearly when reduced to on-page size (see Figure 1).
3. The symbols used for plotting data points should not be too similar, please use different mix of symbols (including open symbols if data points are closely spaced).
4. Symbols should be chosen from the following set if possible.
○ ● △ ▲ ▽ ▼ ◆ ♦ ×
5. Lettering/labelling should not be too small after reduction (see Figure 1).
6. When graphs are generated by computer, lines must not show noticeable stepping.
7. Some shading may not reproduce after reduction. Please make shading as 'coarse' as possible. The preferred order to shading of histogram columns is: open (clear), closed (solid), cross-hatched (lines one way), heavily stippled, and other (if required).

The explanation of the symbols and column headings must be given as a key in the Figure itself and not in the Figure legend.

Line Figures should normally have only left and bottom axes; box-style Figures and those using 3-dimensions are not acceptable.

Where the Figure is a composite of more than one graph, experimental record, etc., particular care is needed to minimize the spaces between each part, without overcrowding the entire Figure.

Figure 1 illustrates a simple properly-drawn graph in its original form (a) and in its reduced form (b) as it would appear in the Journal (single column width).

Photographs and photomicrographs

These should be submitted, twice as large as their intended published size, as good quality prints of high contrast especially where traces and records are illustrated. When

720

Instructions to Authors

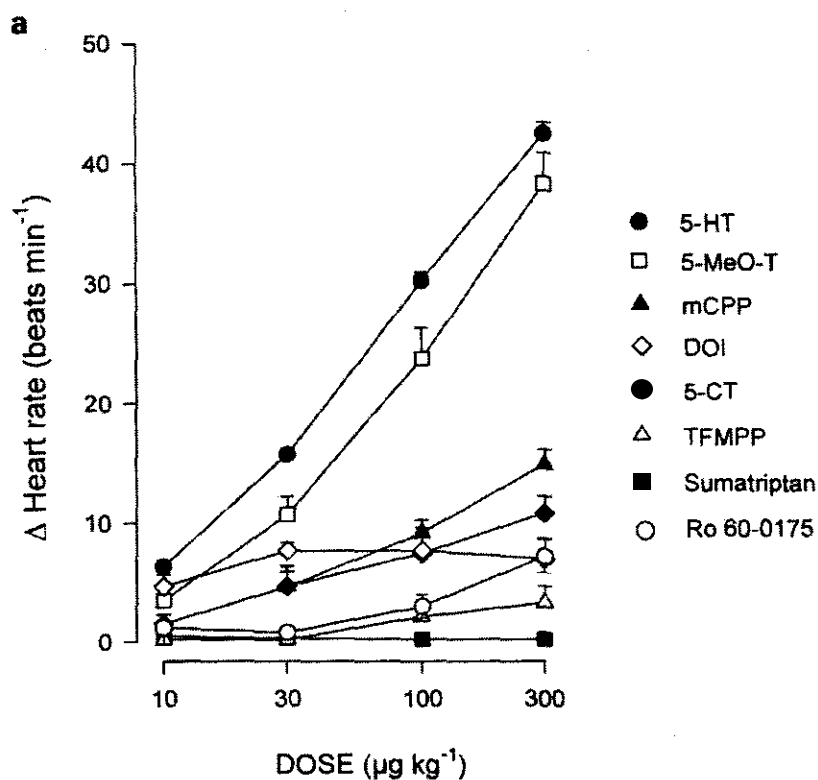


Figure 1

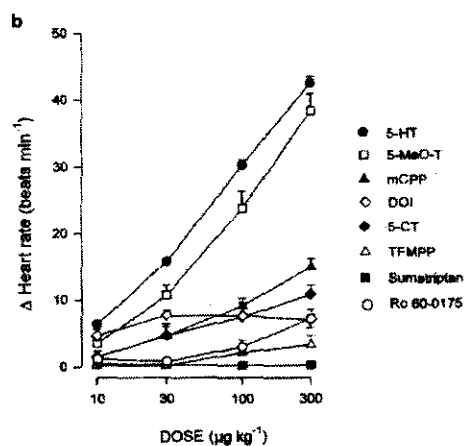


Figure 1

submitting half-tone illustrations for publication authors should remember that it is not possible to reproduce Figures to a finer quality than the original photographs/photomicrographs provided. A CALIBRATION BAR MUST BE PROVIDED ON THE PHOTOMICROGRAPH to ensure that, if the Printer reduces the plate, the scale is reduced in the correct proportion.

Proofs

One set of page proofs are usually sent electronically as e-mail attachments to the corresponding author for checking. The proofs plus any minor corrections must be returned to the Production Controller by fax or post within 48 hours of receipt. Failure to do this will result in delays to the publication. A photocopy of the corrections should be retained by the author.

SPECIAL REPORTS

The purpose of *Special Reports* is to provide rapid publication for new and important results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission, and to state briefly in the covering letter why they believe their work deserves priority publication.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Publisher within 48 hours of receipt**. Authors should ensure that their submitted material conforms exactly to the following requirements.

Special Reports must occupy no more than four printed pages. One copy of the manuscript plus a copy on disk should be provided. The manuscript should be prepared in a two-column format as follows. Text (divided into Summary, Introduction, Methods, Results, Discussion, Acknowledgements and References, maximum of 20, all sub-headings in bold) should be justified and in Times New Roman (9 pt). Columns should measure no more than 83 mm (width) and 250 mm (length). The Title (18 pt bold). Authors (12 pt bold) and their Affiliation (9 pt) should spread across both columns. The Summary (9 pt) should measure 140 mm (width) and should not exceed 150 words (plus Keywords and Abbreviations as for Full Papers). Within this format there is no limitation to the number of illustrations (Figures and Tables, with legends) allowed. Authors must supply illustrations which are appropriately sized for direct inclusion in the Journal. These should be either 80 mm width (including axis labelling) to fit single column or 160 mm width (including axis labelling) to fit double column. Authors must ensure that sufficient space is left in the body of the text for these illustrations with their associated legends. All lettering in Figures should be in plain sans serif typeface (Univers, 8 pt). **Please note that the complete manuscript (including text, Figures and Tables prepared as above) must not exceed the four printed page maximum. Manuscripts which do not conform to these requirements may be returned to authors for amendment without review.**

REVIEW ARTICLES

Authors of review articles, whether unsolicited or commissioned, should first submit a title and short summary (up to 500 words) or its scope to a Senior Editor for approval, in principle. Upon initial approval of short summary the Review should be submitted in the following format:

A summary of 250 words is required.

Up to 8 keywords should be included on the same page as the non-standard abbreviations.

The Review should be between 5000–8000 words.

Sub headings to be in italics and should not be numbered.

Authors are encouraged to include diagrammatic material or published data records as appropriate.

VIDEO SUBMISSION

The preferred format for video submission is Quicktime.

SUBMISSION STATEMENT AND AUTHOR LICENCE

Submission statement

Submission of a manuscript will be taken to indicate the following:

- that Authors have obtained permission to publish from their employers or institutions, if they have a contractual or moral obligation to do so;
- that approvals are held from any persons acknowledged, or cited as having provided personal communication;
- that all authors have seen and approved the final version of the submitted paper;
- that the content of the manuscript is original and that it has not been published or accepted for publication, either in whole or in part, in any form and
- that no part of the manuscript is currently under consideration for publication elsewhere.

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The British Pharmacological Society requires that when a manuscript is accepted for publication in the Journal, all Authors sign the licence granting the British Pharmacological Society rights in publishing the article (**fax and electronic signatures are not acceptable**). This indicates that all authors have seen and approved the final accepted version of the work. The British Pharmacological Society in turn licences Nature Publishing Group to publish the article on behalf of the Society.

The licence is shown at the end of these instructions.

ABBREVIATIONS AND SYMBOLS

Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixes for multiples of units should be used:

Multiplier	Prefix	Symbol
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a
10^3	kilo	k
10^6	mega	M
10^9	giga	G
10^{12}	tera	T

Thus, micron = μm ; ångström = 0.1 nm. Mixed prefixes are not permissible, thus $\text{m}\mu\text{g}$ should be ng . The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbols indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on that line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. P_{A, CO_2} denotes partial pressure of CO_2 in alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

Authors should also consult *Nomenclature Guidelines for Authors* contained in this issue of the Journal. The abbreviations listed may be used without definition. Other chemicals, drugs, enzymes and units must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

Use abbreviations sparingly as extensive use can make the text hard to follow.

Chemical and biological abbreviations

about	~
acetylcholine	Ach
acetylcholinesterase	AchE
acetyl Coenzyme A	Acetyl CoA
adenosine diphosphate	ADP
adenosine monophosphate	AMP
adenosine triphosphate	ATP
adenosine triphosphatase	ATPase
adenosine 3'5' cyclic monophosphate	cAMP
adenyl cyclase	AC
agonist potency	pA_2
alternating current	a.c.
γ -aminobutyric acid	GABA
amino-3-hydroxy-5-methylisoxazole-4-propionic acid	AMPA
analysis of variance	ANOVA
anhydrous	anhyd.
angiotensin converting enzyme	ACE
antagonist potency	pD_2
approximate(ly)	approx.
approximately equals	$\approx$
artificial cerebrospinal fluid	aCSF
blood pressure	BP
bovine serum albumin	BSA
cardiovascular system	CVS
catechol-O-methyl transferase	COMT
central nervous system	CNS
centrifugal force	g
cerebrospinal fluid	CSF
choline acetyltransferase	ChAT
coenzyme A	CoA
complementary deoxyribonucleic acid	cDNA

Use of solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g. mg kg^{-1} rather than mg/kg ; $\text{pmol mm}^{-2} \text{ min}^{-1}$ rather than $\text{pmol/mm}^2/\text{min}$.

Physico-chemical quantities

Quantity	Preferred Unit	Symbol
Amount of substance	mole	mol
Capacitance	Farad	F
Concentration	moles per litre	M or mol l^{-1}
Current	Ampere	A
Electrical conductance	Siemens	S
Electromotive Force	Volt	V
Flow (blood or other liquid)	Litres per second (or min)	l s^{-1} or l min^{-1}
Flow (air or other gas)	Litres per second (or min)	l s^{-1} or l min^{-1}
Force	Newton	N
Frequency	Hertz	Hz
Length	meter	m
Molecular Weight	Dalton	Da
Power	Watt	W
Pressure	Pascal	Pa
Radioactivity	Becquerel	Bq preferred to Ci (3.7×10^{10} Bq)
Resistance	Ohm	Ω
Weight	gram	g

complementary ribonucleic acid	cRNA
correlation coefficient	r
cyclooxygenase	COX
day(s)	d
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D.
dextro-(optical rotation)	(+)
diacylglycerol	DAG
3,4-dihydroxyphenylamine	DOPA
3,4-dihydroxyphenylthylamine	dopamine
direct current	d.c.
disintegrations per minute	dpm
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
edition	edn
editor(s)	ed.
effective concentration 50% of maximum response	EC_{50}
effective dose 50% of maximum response	ED_{50}
electrocardiogram	ECG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	ESR
end plate potential	cpp
equilibrium constant	K
ethylenediaminetetraacetic acid	EDTA
ethylene glycol-bis (β -amino ethyl ether) tetraacetic acid	EGTA
excitatory postsynaptic current	epsc
excitatory postsynaptic potential	epsp

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extracellular fluid	ECF	monoamine oxidase
GABA transaminase	GABA-T	MAO
gas-liquid chromatography	GLC	nitric oxide
glomerular filtration rate	GFR	nitric oxide synthase
glutamic acid decarboxylase	GAD	N-methyl-D-aspartate
guanosine triphosphate	GTP	non-specific binding
guanosine 5'-3'-thio-triphosphate	GTP γ S	nuclear magnetic resonance
guanosine 3'-5' cyclic monophosphate	cGMP	number
guanylyl cyclase	GC	number of observations (statistics)
haemoglobin	Hb	optical density
half-life	$t_{1/2}$	oral
high performance liquid chromatography	HPLC	page/pages
hour or hours	h	p./pp
human serum albumin	HSA	parts per million
hydrogen-ion concentration	[H $^{+}$]	per cent
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH	phenylethanolamine N-methyltransferase
6-hydroxydopamine	6-OHDA	phosphatidyl inositol
N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]	HEPES	phosphodiesterase
5-hydroxytryptamine	5-HT	phospholipase (A $_2$, C, D)
immunoglobulins	IgA; IgD; IgE; IgG; IgM	polymerase chain reaction
inhibitor constant	K $_i$	probability (significance level in a statistical test)
inhibitory concentration (e.g. 50% inhibition of maximum)	IC $_{50}$	protein kinase (A, B, C, G)
inhibitory postsynaptic current	ipsc	PK (A, B, C, G)
inhibitory postsynaptic potential	epsp	radioimmunoassay
international unit	iu	renal blood flow
intra-arterial	i.a.	ribosomal RNA
intracellular fluid	ICF	revolutions per minute
intradermal	i.d.	ribonucleic acid
intramuscular	i.m.	second or seconds
intraperitoneal	i.p.	somatostatin
intracerebroventricular	i.c.v.	standard deviation (of observed sample)
intrathecal	i.t.	standard error (of estimate mean value)
intravenous	i.v.	subcutaneous
isotope (atomic mass) e.g. iodine-131	^{131}I	sum (statistical):
isotopically substituted compounds e.g.	[^{14}C]-ethanol	of hypothetical population
laevo-(absolute configuration)	L-	of observed sample
laevo-(optical rotation)	(-)	thin layer chromatography
lethal dose (in 50% of population)	LD $_{50}$	time, clock 24 h clock used e.g. 18 h 30 min
lipoygenase	LOX	time constant
magnetic resonance imaging	MRI	2-amino-2-hydroxymethyl-propan-1,3,- diol
maximum	max.	transfer ribonucleic acid
mean arterial pressure	MAP	tyrosine hydroxylase
mean value of (statistics)	$\bar{x}$	tyrosine kinase
messenger ribonucleic acid	mRNA	ultraviolet
Michaelis constant	K $_M$	unit
miniature end plate potential	m.epp	volume by volume
minimum	min.	wavelength
minute or minutes	min	weight
		weight by volume



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NOMENCLATURE GUIDELINES FOR AUTHORS

With effect from February 2003

The Nomenclature Working Party (NWP) of the Editorial Board of the British Journal of Pharmacology has consulted many acknowledged experts in an effort to clarify and standardise receptor and other nomenclature systems for use by Editors until a complete set of recommendations from the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) is published.

The NWP is unanimous in its view that with rare exceptions, the Journal should use spellings, names and abbreviations that have been chosen by international bodies convened for the purpose.

For receptor nomenclature, with few exceptions, the Journal generally follows the guidelines laid down in three sources, the current *Trends in Pharmacological Sciences* Nomenclature Supplement, the IUPHAR Receptor Compendia and the reports of NC-IUPHAR Sub Committees published in *Pharmacological Reviews*. In the following Sections, these sources will be referred to as *TIPS Suppl* 12th Edition 2001, *IUPHAR* and *Pharmacol. Rev.*

1 Definition of receptors and subtypes

Receptors and their subtypes are defined in terms of structural information where available, affinity and selectivity of antagonists and thereafter by agonist selectivity.

2 Format of receptor names

It was agreed that, until NC-IUPHAR or *TIPS* provides full recommendations:

- (a) Editors will permit with reluctance new nomenclature systems in papers accepted for publication if, and only if, there are compelling reasons to introduce a new terminology (or modify an accepted one). The criteria upon which the new receptor type or subtype are defined must be given, together with adequate explanations of the relationship between the previous nomenclature (fully referenced) and the proposed one.

N.B. The new nomenclature should not appear in the Title, Short Title or Keywords, unless qualified by the adjective putative (e.g., putative mediated by the putative imidazoline I₂ receptor).

- (b) Only well-established and universally accepted subtype names will be acceptable without any reference to the originator of these terms. In cases of controversy concerning further subdivision of the subtype, full referencing must be given.
- (c) When receptors are expressed from DNA or RNA that has been introduced into cells and these receptors display a dissimilar pharmacological profile to the native receptors, or have not been characterised pharmacologically, they should be denoted by use of lower case, e.g. 5-HT₅ for expressed

receptors and 5-HT₇ for native receptors. The stoichiometry of the expressed receptor should be indicated, where appropriate, e.g. for an adult muscle nicotinic acetylcholine receptor, it might be (α_1)₂ $\beta_1\gamma\delta$.

- (d) Receptor subtypes should be designated by means of a subscript numeral or capital letter. Some double subscripts (i.e. numeral plus letter) are acceptable.
- (e) Greek letters and Roman numerals should be avoided in any new nomenclature. The name should not include the letter 'R' or 'r' as an abbreviation for receptor.
- (f) Mammalian systems are the basis of receptor classifications with primacy given to humans. Therefore non-mammalian species should be clearly indicated, e.g. *Torpedo* nicotinic acetylcholine receptor, turkey β adrenoceptor, locust GABA receptor.

3 Types of receptor

The NWP accepts that there are additional receptors to those described below which can be considered to be well characterised. In many cases, however, their existence has been confirmed only in cloning studies and it is as yet unclear how they relate to similar subdivisions proposed on the grounds of differences in agonist and antagonist potencies in various tissues. Italics should be used if the receptor has not been cloned. Lower case should be used to describe cloned receptors for which endogenous expression has not been described. It is incorrect to refer to any receptor, using the suffix 'ergic' (e.g., cholinergic, adrenergic). See Section 5 for the correct use of such terms.

- (1) *Acetylcholine receptors* The two principal subfamilies are muscarinic and nicotinic acetylcholine receptors.

Muscarinic acetylcholine receptors The principal subtypes are M₁, M₂, M₃, M₄ and M₅. (See Caulfield, M.P. and Birdsall, N.M.J. (1998) *Pharmacol. Rev.*, 50, 279–290.

Nicotinic acetylcholine receptors Receptors should be described as nicotinic (not n), and where it is known the stoichiometry should be given at first mention. NC-IUPHAR recommend that nicotinic receptor subtypes should be classified according to the predominant α subunit (α_1 – α_{10}) contained within the receptor complex. (See Lukas, R.J. *et al.*, (1999) *Pharmacol. Rev.*, 51, 379–401).

- (2) *Adenosine receptors* Principal subtypes are A₁, A_{2A}, A_{2B} and A₃. (See Fredholm, B.B. *et al.*, (2001) *Pharmacol. Rev.*, 53, 527–522).

- (3) *Adrenoceptors* The principal subtypes are α_1 -, α_2 -, β_1 -, β_2 - and β_3 -adrenoceptors. Additional subtypes

must be fully referenced. (See Bylund, D.B., *et al.* (1994) *Pharmacol. Rev.*, **46**, 121–136 and Hieble, J.P., *et al.* (1995) *Pharmacol. Rev.*, **47**, 267–270).

For further subdivision, please refer to Bylund, D.B., *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 88–103 IUPHAR media, London.

- (4) **Angiotensin receptors** The principal subtypes are AT₁ and AT₂. (See de Gasparo M., *et al.* (2000) *Pharmacol. Rev.*, **52**, 415–472).
- (5) **Atrial natriuretic peptide receptors** The principal subtypes are ANP_A and ANP_B.
- (6) **Bombesin receptors** The principal subtypes are BB₁, BB₂ and BB₃.
- (7) **Bradykinin receptors** The principal subtypes are B₁ and B₂.
- (8) **Calcitonin gene-related peptide (CGRP) receptors** The principal subtypes are calcitonin (CT), Amylin (AMY), CGRP, and adrenomedullin (AM). (See Poyner, D.R. *et al.* (2002) *Pharmacol. Rev.*, **54**, 161–202).
- (9) **Cannabinoid receptors** The principal subtypes are CB₁ and CB₂.
- (10) **Chemokine receptors** The principal subgroups are XCR, CCR, CXR, CXCR, CX₃CR. Subtypes within these subgroups must be fully referenced (See Murphy, P.M., *et al.* (2000) *Pharmacol. Rev.*, **52**, 145–168).
- (11) **Cholecystokinin (CCK) receptors** The principal subtypes are CCK₁ and CCK₂. (See Noble, F., *et al.* (1999) *Pharmacol. Rev.*, **51**, 745–781).
- (12) **Corticotrophin receptors** The principal subtypes are CRF₁ and CRF₂.
- (13) **Dopamine receptors** The principal subtypes are D₁, D₂, D₃, D₄ and D₅. (See Schwartz J.-C., *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 171–181, IUPHAR media, London).
- (14) **Endothelin receptors** The principal subtypes are ET_A and ET_B. (See Davenport, A.P. (2002) *Pharmacol. Rev.*, **54**, 219–226).
- (15) **Galanin receptors** The principal subtypes are GAL₁, GAL₂ and GAL₃. (See Branchek, T. *et al.* (2000) *Trends Pharmacol. Sci.*, **21**, 109–116).
- (16) **γ-Aminobutyric acid (GABA) receptors** The principal subtypes are GABA_A and GABA_B. Modulatory sites on either receptor type should be fully referenced. (See Barnard, E.A. *et al.* (1998) *Pharmacol. Rev.*, **50**, 291–313; Bowers, N.G. *et al.* (2002) *Pharmacol. Rev.*, **54**, 247–264).
- (17) **Glucagon like peptide receptors** The principal subtypes are GLP₁ and GLP₂.

(18) **Glutamate receptors** Three ionotropic subtypes are recognised and named: (1) NMDA receptors; (2) AMPA receptors, and (3) kainate receptors. The metabotropic glutamate (mGlu) receptors are subdivided into mGlu_{1,2,3,4,5,6,7,8}. (See Schoepp, D.D., *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 195–206, IUPHAR media, London). Modulatory sites must be fully referenced.

(19) **Glycine receptor** When it is known the stoichiometry should be given at first mention. A distinction should be drawn between the immature receptor composed of α₂ subunits expressed by embryonic neurons and the mature receptor containing predominantly α₁ and β subunits expressed by adult neurons.

(20) **Histamine receptors** The principal subtypes are H₁, H₂, H₃ and H₄ (See Hill, S.J. (2000)) *IUPHAR compendium of receptor characterisation and classification* pp. 227–232, IUPHAR media, London; *TiPS Suppl* 12th Edition, 2001, p. 60.)

(21) **5-Hydroxytryptamine (5-HT) receptors** The principal subtypes are 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇. Further subdivisions have been defined (See Martin, G.R. *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 233–251, IUPHAR media, London).

(22) **Inositol (1,4,5)-trisphosphate receptor (Ins(1,4,5)P₃)** The principal subtypes are IP₃1, IP₃2 and IP₃3.

(23) **Leukotriene (LT) receptors** The principal subtypes are BLT₁, BLT₂, CysLT₁ and CysLT₂. Further leukotriene receptor subtypes should be fully referenced.

(24) **Lysophospholipid (LPL) receptors** The principal subtypes are LPA₁, LPA₂, LPA₃, and SIP₁, SIP₂, SIP₃, SIP₄, SIP₅ (See Chun, J. *et al.* (2002) *Pharmacol. Rev.*, **54**, 265–269).

(25) **Melanocortin receptors** The principal subtypes are MC₁, MC₂, MC₃, MC₄ and MC₅.

(26) **Melatonin receptors** The principal subtypes are MT₁, MT₂ and MT₃. (See *TiPS Suppl*, 12th Edition, 2001, p. 72).

(27) **Neuropeptide Y receptors** The principal subtypes are Y₁, Y₂, Y₄, Y₅ and y₆. (See Michel, M.C. *et al.* (1998) *Pharmacol. Rev.*, **50**, 143–150).

(28) **Neurotensin receptors** The principal subtypes are NT₁ & NT₂.

(29) **Neurotrophin receptors** The principal subtypes are trkA, trkB, trkC and p75. (See *TiPS*, 12th Edition, 2001, p. 75).

(30) **Opioid and Opioid-like receptors** The principal subtypes are δ (DOP), κ (KOP), μ (MOP) and ORL1 (NOP). Other proposed subtypes should be fully referenced.

- (31) *Peroxisome proliferator activated receptors* The principal subtypes are PPAR- α , PPAR- β and PPAR- γ .
- (32) *Prostanoid receptors* The principal types are DP, EP, FP, IP and TP. When first mentioned, the style prostanoid (XP) receptor should be used, thereafter, XP receptor (where X denotes the type). Proposed subtypes should be referred to as XP_n (e.g. EP₁, EP₂) and referenced (See Coleman, R.A. *et al.* (1994) *Pharmacol. Rev.*, **45**, 205–229; Coleman, R.A. *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 337–353, IUPHAR media, London).
- (33) *Protease activated receptors* The principal subtypes are PAR1, PAR2, PAR3 and PAR4. (See Hollenberg, M. D. and Compton, S.J. (2002) *Pharmacol. Rev.* **54**, 203–217).
- (34) *P2X receptors* The principal subtypes are P2X_{1,2,3,4,5,6,7} (See Khakh, B.S. *et al.* (2001) *Pharmacol. Rev.* **53**, 107–118).
- (35) *P2Y receptors* The principal subtypes are P2Y_{1,2,4,6,11,12,13}. (See King, B.F. *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 306–320, IUPHAR media, London; *TiPS*, 12th Edition, 2001, 80–82).
- (36) *Ryanodine receptors* The principal subtypes are RY₁, RY₂ and RY₃. (See *TiPS*, 12th Edition, 2001, p. 91).
- (37) *Somatostatin (SST) receptors* The principal types are SRIF₁ and SRIF₂. Proposed subtypes should be fully referenced. (See Hoyer, D. *et al.* (2000) *IUPHAR compendium of receptor characterisation and classification*, 2nd Edition, pp. 354–364, IUPHAR media, London).
- (38) *Tachykinin (Neurokinin-NK) receptors* The principal subtypes are NK₁, NK₂ and NK₃. (See *TiPS*, 12th Edition, 2001, p. 94).
- (39) *Vanilloid receptor* The principal subtype is TRPV1 (formerly VR1). Other proposed subtypes should be fully referenced.
- (40) *Vasoactive intestinal peptide and pituitary adenylate cyclase activating peptide receptors* The principal subtypes are VPAC₁, VPAC₂ and PAC₁. (See Harmar, A.J. *et al.* (1998) *Pharmacol. Rev.*, **50**, 265–270).
- (41) *Vasopressin and oxytocin receptors* The principal subtypes are V_{1A}, V_{1B}, V₂ and OT. Further subtypes should be fully referenced.

4 Naming of ion channels

Ion channels are typically described by an abbreviation of the ion permeating the channel (e.g. K⁺ channel, Na⁺ channel, Cl⁻ channel, Ca²⁺ channel etc.) Ionic currents are referred to by either the full description of the current (e.g. Ca²⁺-activated K⁺ current) or as an abbreviation using the prefix *I* followed by the atomic species carrying the current as a subscript (e.g. *I*_{Na}, *I*_{Ca}). Where it is

important to specify the activator of the current, this may be added to the atomic species subscript in parenthesis (e.g. *I*_{K(Ca)} for a calcium-activated potassium current or *I*_{K(V)} for a voltage-activated potassium current).

When a pharmacological agent is used to describe a current, it must be fully defined in the text.

When, for example, a system has two currents carried by the same ionic species and activated by similar means but with (for example) different kinetics, the distinguishing factor may be added to the subscript or as a hyphen after the abbreviation (e.g. '*I*_{K(Ca,slow)}' or '*I*_{K(Ca)-slow}', which would refer to a kinetically slow calcium-activated potassium current).

Examples of some commonly used abbreviations are shown below:

Sodium current: *I*_{Na(V)}
Potassium currents: *I*_{K(V)}, *I*_{K(Ca)}, *I*_{K(A)}, *I*_{K(IR)}
Chloride currents: *I*_{Cl(Ca)}, *I*_{Cl(AMP)}, *I*_{Cl(swell)}
Calcium currents: *I*_{Ca(L)}, *I*_{Ca(T)}, *I*_{Ca(N)}

5 Naming of nerve fibres

Many nerve fibres are now known to release more than one transmitter, and future work may show that this is, in fact, the general rule. In that case, the concept of the same transmitter being released either at different developmental stages or under various experimental conditions would no longer hold, and single adjectives that imply this (e.g. cholinergic, noradrenergic) would become inappropriate when applied to nerve fibres, as distinct from transmitter functions. For the present, those nerve fibres that are known to function by releasing more than one identified transmitter may be described accordingly; for example, cholinergic-peptidergic (in alphabetical order, the order implying no priority of function). N.B. The suffix 'ergic' should continue to be applied only to nerve fibres and to the transmission event, in accordance with Dale's intentions. For example 'cholinergic' indicates that the nerve fibre, or the transmission, functions through the release of a choline like substance. The suffix should not be used loosely to mean 'pertaining to'. Hence the expression 'cholinergic receptor' (rather than acetylcholine receptor) is incorrect. Transmission events involving nitric oxide may be referred to as *nitrergic*.

Nerve fibres that release noradrenaline are to be described as noradrenergic. The term adrenergic should be reserved for nerve fibres known to release adrenaline. Where the identity of the catecholamine is uncertain catecholaminergic should be used. The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic).

NANC is an acceptable abbreviation of non-adrenergic, non-cholinergic for peripheral efferent nerve fibres when the identity of the transmitter(s) is unknown.

Glutamatergic, not glutaminergic, should be used to describe nerve fibres releasing glutamate. In referring to peptide-releasing nerve fibres (e.g. those that may release substance P or vasoactive intestinal peptide) the nomenclature to be used is peptidergic (X), e.g. peptidergic (SP).

The terms 5-hydroxytryptamine (5-HT) and 5-hydroxytryptaminergic (i.e. nerves releasing 5-hydroxytryptamine) are preferred to those of serotonin and serotonergic.

The term 5-HT₂ is not acceptable.

The term purinergic defines transmission mediated by ATP.

6 Terms used to describe agonist and antagonist action

The following terms can be used without full definition. Where appropriate, other terms may be used but must be accompanied by a full definition.

Terms used to describe affinity and potency

(a) *EC₅₀* The concentration of an agonist that produces 50% of the maximal response for that agonist *in vitro*. The agonist may be stimulatory or inhibitory. When *EC₅₀* values are determined in the presence of other agonists or antagonists the concentration of the latter should be stated. Related terms, e.g. *EC₂₅*, are acceptable if accompanied by a full definition.

(b) *IC₅₀* This term may be used in the following ways.

(i) The concentration of antagonist that reduces the response to a sub-maximal concentration of agonist by 50%; the concentration of agonist should be stated.

(ii) The concentration of competing agonist or antagonist that inhibits the binding of a radioligand by 50%; the concentration of radioligand should be stated.

(c) *ED₅₀* This term may be used in the following ways.

(i) The dose of an agonist or antagonist that produces 50% of the maximal possible effect of that agonist or antagonist *in vivo*.

(ii) The dose of drug that produces the effect under investigation in 50% of the population.

(d) *K* The dissociation equilibrium constant (mol l^{-1}), for ligand receptor interactions. The reciprocal is called the affinity constant or association equilibrium constant. When necessary for clarity, subscripts (letters or numerals, or a combination of both) may be added but these must be clearly explained when first used.

(e) *n_H* The Hill coefficient.

(f) *pA₂* The negative logarithm to base 10 of the concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit a given submaximal response. Note that the definition is empirical and does not pre-suppose the mechanism of antagonism. The *pA₂* value can be determined from a Schild plot with unconstrained slopes, but only provides an estimate of the *pK_B* if the antagonism has been shown to meet all of the criteria of competition.

(g) *pD₂* The negative logarithm to base 10 of the *EC₅₀*.

(h) *pIC₅₀* The negative logarithm to base 10 of the *IC₅₀*.

(i) *pK* The negative logarithm to base 10 of *K* (with or without subscripts as appropriate: see under *K* above).

Terms used to describe the mode of antagonism

(a) *Competitive antagonism* In competitive antagonism the binding of agonist and antagonist is mutually exclusive. This may be because the agonist and antagonist compete for the same binding site or combine with adjacent sites that overlap. A third possibility is that different sites are involved but they influence the receptor macromolecule in such a way that agonist and antagonist molecules cannot be bound at the same time.

(b) *Irreversible competitive antagonism* Used to describe antagonists that bind irreversibly.

(c) *Non-competitive antagonism* Agonist and antagonist can be bound simultaneously; antagonist binding reduces or prevents the action of the agonist.

(d) *Un-competitive antagonism* Antagonist binding is dependent upon prior agonist activation (e.g. open channel blockade).

(e) *Irreversible non-competitive antagonism* Used to describe non-competitive antagonists that bind irreversibly.

For a more detailed account of the terms used to describe agonist and antagonist action see Jenkinson, D.H., *et al.* (1995) *Pharmacol. Rev.*, 47, 225–266.

7 Guidance on statistical analysis of data

Given that populations of data with a Gaussian (normal) distribution on a logarithmic scale (e.g. *pD₂*, *pEC₅₀*, etc.) will show a skewed distribution once converted to a natural scale, parametric analysis is only appropriate on the logarithmic scale for such data. Thus, the expression of *EC₅₀* and *ED₅₀* values (derived from log concentration- or log dose-response curves) as means $\pm$ s.e.mean is inappropriate. However, *pD₂* or *pIC₅₀* values may be reported as means $\pm$ s.e.mean.

8 Enzymes

The International Union of Biochemistry and Molecular Biology Enzyme Commission (EC) number and full name (Enzyme Nomenclature 1992, Academic Press, San Diego and London) must be quoted when first mentioned in text. Subsequently the accepted trivial name is used. Trivial names may be used in the title. The following abbreviations may be used; cyclo-oxygenase (COX-1 and COX-2), guanylyl cyclase (GC), monoamine oxidase (MAO-A and MAO-B), nitric oxide synthase (eNOS, nNOS and iNOS), phosphodiesterase (PDE1 to PDE11), catechol-O-methyl transferase (COMT), adenylyl cyclase (AC), acetylcholinesterase (AChE), acetyl coenzyme A (AcetylCoA), adenosine triphosphatase (ATPase), angiotensin converting enzyme (ACE), choline acetyltransferase (ChAT), coenzyme A (CoA), deoxyribonuclease (DNase), GABA transaminase (GABA-T), glutamic acid decarboxylase (GAD), lipoxygenase (LOX), phenylethanolamine N-methyltransferase (PNMT), phospholipase (PLA₂, PLC, PLD), protein kinase (PKA, PKB, PKC, PKG), tyrosine hydroxylase (TH), tyrosine kinase (TK), ribonuclease (RNase).

Nomenclature Guidelines

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9 Transporters

Transporters should be defined in full at first mention.

10 Other nomenclature requirements

- (a) *Racemates* Authors must state unambiguously in the Methods section of papers which isomers were used, e.g. (+)- or (–)-propranolol, and must bring to the attention of the reader the composite character of drugs that are mixtures of stereoisomers. Furthermore, the implications of the composite nature of such drugs studied for the interpretation of the data measured and the conclusions drawn must be made explicit. Capital R and S refer to the absolute configurations of chiral centres and should be used where necessary.
- (b) *Purines* This term should not be used as a synonym for purine nucleotides or nucleosides.
- (c) *Eicosanoids* The system of nomenclature to be used for eicosanoids is that published in *Methods in Enzymology* (1990) 187, 1–9. In manuscripts, the first use of the full chemical name of any eicosanoid

should indicate double bond geometry when this is known.

- (d) *Cell lines* Cell type, range of passage number, species and source should be defined.
- (e) *Molecular biology* Abbreviations pertaining to molecular biological techniques need to be defined or presented in such a way that they can be recognised by the non-specialist.
- (f) *Tension* Tension is force and should be calibrated in Newtons. (1 Newton = 1 kg m s^{–2}) or in kg weight, g weight, or mg weight etc. It should not be calibrated in units of mass (e.g. kg). (See Miller D.J. (1988) *Trends Pharmacol. Sci.*, 9, 124–5).
- (g) *Ions* When referring to ions, the charge should be indicated, e.g. Na⁺, Ca²⁺, 3Na⁺/Ca²⁺ exchange, etc.
- (h) *Inhibitors of nitric oxide synthase (NOS)* Commonly used inhibitors of NOS and their appropriate abbreviations include L-N^G-mono-methylarginine (L-NMMA), L-N^G-nitroarginine (L-NOARG), and L-N^G-nitroarginine methyl ester (L-NAME).

***Instructions to the Authors:
Molecular Pharmacology
(Appendix to Chapter 4)***

**Appendix
C**

A copy of the "Instructions to the Authors" of *Molecular Pharmacology* follows on the next page.

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Appendix
D

A copy of the "Instructions to the Authors" of *British Journal of Clinical Pharmacology* follows on the next page.

British Journal of Clinical Pharmacology

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1 Davies RH, Sheridan DJ. The treatment of heart failure—what next? *Br J Clin Pharmacol* 1993; 35:557–563.

2 Hoffman BB, Lefkowitz RJ. Beta-adrenergic receptor antagonists. In *The Pharmacological Basis of Therapeutics*, Eighth Edition, eds Gilman AG, Rail TW, Nies AS, New York: Pergamon Press, 1990: 229–243.

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Contributions to Conferences**Appendix
E*****E.1 Poster presentations***

Title: *Non-specific mechanisms of antagonism by the classical irreversible competitive antagonist benextramine on α_{2A} -adrenergic receptors*

Authors: *Brink, C.B.*, Bodenstein, J. & Venter, D.P.*

Presenter: *Bodenstein, J.*

Institution: *Division of Pharmacology, Potchefstroom University for CHE, Potchefstroom, South Africa (*e-mail: fklcbb@puknet.puk.ac.za)*

Conference: *International South African Immunology and Pharmacology Congress; Sun City, South Africa; 16 – 20 September 2001*

Abstract: Irreversible competitive antagonists, such as phenoxybenzamine and benextramine, are generally assumed to selectively and irreversibly block specific receptors. There are several reports and queries, however, suggesting that these drugs may not be selective for binding to specific receptors, thereby also affecting responses via other mechanisms.

The aim of this study was to investigate proposed non-specific mechanisms of antagonism by the classical irreversible competitive α_2 -adrenergic receptor antagonist benextramine.

A series of experiments was conducted utilising a CHO-K1 cell line that was genetically manipulated to express high numbers of the porcine α_{2A} -adrenergic receptors. Ligand-binding studies and dose-response curves of the full agonist UK 14,304 were performed after the cells were treated with 0, 1, 10 and 100 μ M of benextramine for 20 minutes, with or without receptor protection by a high concentration (10 μ M) of the competitive antagonist yohimbine.

Results indicate that, at higher concentrations, benextramine also binds to non-specific binding sites, influencing response. The implication of this finding is that benextramine cannot be used indiscriminately to estimate agonist efficacy, utilising the Furchgott analysis. Scientists should be careful not to build conclusions upon such assumptions.

Title: *Ethical reflections on genetic manipulation: A normative perspective on interspecies (e.g. human-animal) transfer of genetic material for research*

Authors: *Bodenstein, J.* & Brink, C.B.*

Presenter: *Bodenstein, J.*

Institution: *Division of Pharmacology, Potchefstroom University for CHE, Potchefstroom, South Africa (*e-mail: fkljb@puknet.puk.ac.za)*

Conference: *International South African Immunology and Pharmacology Congress; Sun City, South Africa; 16 – 20 September 2001*

Abstract: The use of genetically manipulated cells, tissues and animals are common practise in current biological research. There are numerous advantages for using genetically manipulated cells, allowing us to control our experimental conditions optimally in order to find new answers to pending scientific questions. However, ethical dilemmas are also created by new technology. Genetic material can be transferred between species (including humans and animals) in order to create "new" organisms/forms of life. Worldviews are now challenged with new ethical dilemmas: What is the essence of life? How far may scientists go to expand horizons and abilities responsibly?

The aim of this study was to gain some insight into the relevant ethical questions and debate regarding genetic manipulation. Literature searches were conducted, utilising the Internet and other resources available.

As can be expected, debates by biologists and ethicists subscribing to different worldviews was found to have markedly different suppositions, reasoning and answers to this important dilemma. In order to maintain a responsible approach to research and technology, most worldviews, including the Christian worldview, need to thoroughly reflect on the meaning of life and in particular of human life anew.

Title: *Non-competitive irreversible antagonism by phenoxybenzamine and benextramine on α_{2A} -adrenergic receptors*

Authors: *Brink, C.B.*, Bodenstein, J. & Venter, D.P.*

Presenter: *Brink, C.B.*

Institution: *Division of Pharmacology, Potchefstroom University for CHE, Potchefstroom, South Africa (*e-mail: fklcbb@puknet.puk.ac.za)*

Conference: *International Union of Pharmacology (IUPHAR) Congress; San Francisco, CA, U.S.A.; 7 – 12 July 2002*

Abstract: Drugs such as phenoxybenzamine and benextramine are examples of classical α_2 -adrenergic receptor irreversible competitive antagonists. There are reports in literature, however, suggesting that such drugs most likely also antagonise agonist response via non-specific mechanisms.

The aim of this study was to investigate proposed irreversible non-specific mechanisms of antagonism by phenoxybenzamine and benextramine at α_{2A} -adrenergic receptors.

A series of experiments were conducted utilising the CHO-K1 cell line expressing the porcine α_{2A} -adrenergic receptor. Ligand-binding studies and dose-response curves with the full agonist UK 14,304 were performed after 20-minute treatment with an irreversible antagonist, with or without receptor protection by a competitive antagonist.

Results clearly indicate that phenoxybenzamine and benextramine exhibit irreversible competitive and non-competitive antagonism. They inhibit the signal-transduction system (metactoid antagonism), without affecting agonist affinity for the receptor. These drugs can therefore not be used indiscriminately to estimate spare receptor fraction, or relative agonist efficacy utilising the classical Furchgott analysis.

E.2 Podium presentations

Title: *Unravelling novel non-competitive mechanisms of action of selected classical irreversible competitive antagonists*

Authors: *Brink, C.B.*, Bodenstein, J. & Venter, D.P.*

Presenter: *Bodenstein, J.*

Institution: *Division of Pharmacology, Potchefstroom University for CHE, Potchefstroom, South Africa (*e-mail: fklcbb@puknet.puk.ac.za)*

Conference: *Third International Conference on Pharmaceutical and Pharmacological Sciences (ICPPS); Boksburg, South Africa; 22 – 25 September 2002*

Abstract: Several reports suggested that classical irreversible competitive antagonists may also exhibit non-specific mechanisms of antagonism, impacting on the interpretation of published data investigating e.g. the relative efficacy of full agonists in systems with spare receptors.

The aim of this study was to investigate the nature of the non-specific mechanisms of antagonism by the classical α -adrenoceptor antagonists phenoxybenzamine and benextramine and the cholinergic muscarinic receptor antagonist 4-DAMP-mustard.

A CHO-K1 cell line expressing the porcine α_{2A} -adrenergic receptor and a human neuroblastoma cell line (SH-SY5Y) endogenously expressing muscarinic receptors, were pre-treated for 20 minutes with different concentrations of the appropriate irreversible antagonist, with or without receptor protection by a high concentration of a reversible competitive antagonist. After rinsing and washing procedures, receptor concentration or agonist affinity values were obtained from appropriate radioligand binding experiments, or functional data (agonist-mediated response) were obtained from measuring second messenger formation or GTP γ S binding to G proteins. Results indicate that the irreversible antagonists also bind to non-specific sites, influencing response via the signal transduction (metactoid) mechanism. The implication of this finding is that these drugs cannot be used indiscriminately to eliminate spare receptors in order to estimate agonist relative efficacy, utilising the Furchgott analysis.

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"For he shall give his angels charge over thee, to keep thee in all thy ways. They shall bear thee up in their hands, lest thou dash thy foot against a stone." — **Psalms 91:11-12 (Holy Bible, King James Version 1611, 1995).**

Father in Heaven

Thank you **Father in Heaven** for all the opportunities, talents, help and good health You have given to me to complete this Ph.D.-study. Without You nothing would have been possible for me to accomplish, for in You all truth, wisdom, and life are found. *Soli deo Gloria.*

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Granter

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