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PHARMACOLOGICAL EVALUATION OF TRIMETOQUINOL ANALOGS
AS AFFINITY LIGANDS FOR β-ADRENERGIC RECEPTOR SYSTEMS

A Dissertation
Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate School of
The Ohio State University

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ABSTRACT

Analogs of the β-adrenoceptor (β-AR) agonist, trimetoquinol (TMQ) possessing potent agonist activity and selectivity at the β₃-AR subtype, may have use in the treatment of obesity, non-insulin-dependent diabetes and gastrointestinal hypermotility disorders. While the catechol portion of the compound is essential for potent agonist activities at the β₁- and β₂-ARs, the 1-benzyl side-chain of TMQ is important for selective activity on the β₃-AR subtype. Characterization of the interactive site for the 1-benzyl ring of TMQ is necessary for rational design of potent and selective β₃-AR agonist analogs. Hence, 4'-substituted analogs including affinity (α-haloacetamido and isothiocyanato) and photoaffinity (azido) analogs of 3'-iodo-5'-demethoxy- or 3',5'-diiodo-TMQ were evaluated for their binding affinities and biochemical potencies (cAMP accumulations) on rat β₃-AR and the three human β-AR subtypes expressed in Chinese hamster ovary (CHO) cells, and for functional activities on isolated rat tissues. These TMQ analogs possessed higher affinities and agonist potencies than (-)-isoproterenol on the β-AR subtypes, and the biochemical potencies were in agreement with values obtained in functional assays. Agonist potencies were comparable to that of the β₃-AR-selective agonist, BRL 37344; however, unlike the latter compound, the TMQ analogs
are full agonists on the human $\beta_3$-AR. 4'-Acetamido-3',5'-diiodoTMQ and 4'-azido-3'-iodo-5'-demethoxyTMQ are among the most potent $\beta$-AR agonists currently known.

Activities of the TMQ derivatives and isoproterenol on human $\beta_2$-AR were blocked by propranolol in a concentration-dependent manner. However, the agonist-dependent discordance in the pA$_2$ values of propranolol on human $\beta_2$AR implies that sites of interaction of the 4'-acetamido- and 4'-$\alpha$-chloroacetamido-3',5'-diiodoTMQ analogs with this receptor differ from that of isoproterenol. Additionally, 4'-$\alpha$-chloroacetamido-3',5'-diiodoTMQ and 4'-isothiocyanato-3'-iodo-5'-demethoxyTMQ demonstrated significant concentration-dependent irreversible binding to the rat $\beta_3$- and human $\beta_2$-AR, respectively. However, the failure of reversible $\beta$-AR ligands to protect against this irreversible binding indicates that the lipophilic 1-benzyl ring of these affinity TMQ analogs interacts with a hydrophobic region of the $\beta$-AR that may represent an "exo-site" or allosteric binding site on the receptor. By contrast, the photoaffinity (azido) TMQ analog demonstrated concentration- and time-dependent irreversible binding to the human $\beta_2$-AR. This was protected by competing $\beta$-AR ligands, implying an overlap of interaction sites of these compounds within the receptor binding domains.

These studies confirm the importance of the 1-benzyl ring of TMQ in agonist interactions with the $\beta$-ARs. Affinity and photoaffinity labels of the 1-benzyl substituted analogs of TMQ may serve as useful tools for characterizing the receptor binding domains of the $\beta$-AR, and thereby provide clues for the design of potent and selective $\beta_3$-AR agonist analogs of TMQ.
Dedicated to my mother
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CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1. Drug Receptor Theory

1.1.1. Emergence of the Receptor Concept

Drugs are broadly defined as chemical substances that cause physical or chemical perturbations in a biological system. Several drugs produce their effects by virtue of their physicochemical properties (e.g. antacids, surfactants, astringents, general and local anesthetics). However, the vast majority of drug compounds are mimics of endogenous hormones, autacoids and neurotransmitters which interact selectively by a lock and key mechanism with certain cellular proteins and initiate or interrupt specific cascades of molecular events in the cells, leading to a physiological response. The endogenous biochemicals and drug compounds are the "key", while the "lock" is constituted generally of cellular proteins with well-defined structural specifications, generally located on the cell membrane, and commonly termed as receptors. Receptor proteins are found in very small amounts but
constitute a significant constituent of the cellular machinery. The receptor concept emerged at the turn of the century as a result of studies by two pioneers. J.N. Langley (1852-1926) studied effects of compounds on autonomic transmission and determined that curare as well as nicotine acted at a common site on frog gastrocnemius muscle (Langley, 1909). Paul Ehrlich (1854-1915) experimented with tissue stains, snake venoms and bacterial toxins. Ehrlich's Nobel prize winning work involving elucidation of antigen-antibody reaction enhanced the concept of cell surface receptors. In his later work with arsenals, he established the concept of binding of small molecules to target cells (Ehrlich, 1913).

1.1.2. The Occupational Theory

The foundation of this theory, which attempts to quantitate drug-receptor interactions, was described by Clark (1885-1941). Based on studies of acetylcholine in frog heart and rectus abdominus muscle, he proposed a mathematical relationship of drug-receptor interactions similar to Langmuir's adsorption isotherm (Langmuir, 1918) which is governed by the law of mass action. According to this theory, the magnitude of pharmacological response was proportional to the number of receptors occupied by the drug and that the receptor population was negligible relative to the total amount of drug present in the medium (Clark, 1926). He also assumed that one molecule of drug interacted with only one receptor, and that maximum response required occupation of all receptors in the tissue. Clark was also the first to recognize that response was a result of two processes: "fixation" or binding of the drug
to receptors, and a second process following the binding (Clark, 1937). Years later, upon observing quantitative differences in activities of members of a homologous series of p-aminobenzoates, Ariëns defined the second process as intrinsic activity ($\alpha$) and quantified this property on a scale where $\alpha = 1$ for full agonists, 0 for antagonists and $0 < \alpha < 1$ for "dualists" or partial agonists (Ariëns, 1954). But, while Ariëns retained Clark's assumptions on receptor occupation, Stephenson (1956) introduced an important modification to the theory by demonstrating that pharmacological response did not need to be linearly related to receptor occupation and that an agonist can produce maximum effect by occupying only a small fraction of receptors. The concept of spare receptors was introduced. He defined the capacity of drugs to elicit a response upon occupying a specific fraction of receptors as efficacy ($e$). Efficacy differs from the intrinsic activity defined by Ariëns, in that two full agonists with $\alpha = 1$, can have different efficacies. Independent studies by Furchgott (1955) and Nickerson (1956) with irreversible receptor antagonists supported Stephenson's modification of occupation theory. Independently, Nickerson also proposed the existence of receptor reserve or spare receptors in tissues.

Furchgott developed specific techniques for determining dissociation constants of agonists in isolated tissues using irreversible antagonists (Furchgott, 1966; Furchgott and Bursztyn, 1967) and contributed significantly to the receptor theory by defining Stephenson's postulate of efficacy as a product of intrinsic efficacy ($e$), which is a drug parameter relating to the ability of the drug molecule to produce a stimulus, and of the concentration
of active receptors, $R_r$ in the tissue (i.e. $e = e_0[R_r]$). Thus, while efficacy is dependent on receptor density in tissues, intrinsic efficacy is an intrinsic property of the drug molecule to induce conformational changes in the receptor. However, limitations of experimental techniques prevent estimation of intrinsic efficacies, and only relative values between different agonists can be determined. The operational model of drug action was introduced by Black and Leff (1983) to simplify quantitation of drug-receptor interactions. While this model is based on the occupancy theory, it replaces intrinsic efficacy and stimulus-response function with a measurable parameter of coupling of agonist-receptor complex with a transducer element (e.g. G-proteins).

1.1.3. The Rate Theory

Paton (1961) observed that generally antagonists act much more slowly than agonists and that their potencies are inversely proportional to their rates of dissociation from the receptors. Also, in guinea pig ileum, a series of alkyltrimethylammonium compounds produced transient peak responses followed by steady-state responses of lower magnitude, which could not be explained by the occupation theory. He proposed that drug action depends upon the rate of association of the molecule with the receptor. It was assumed that each association of drug with receptor resulted in a quantum of excitation and total number of excitation quanta would determine magnitude of response. Compounds which dissociate rapidly from receptors would be able to reassociate more number of times to generate greater quanta of excitation in a given period and therefore act as agonists, whereas competitive
antagonists would be compounds with low dissociation rate constants. The ratio of the association and dissociation rate constants would determine affinity while the dissociation rate constant would determine stimulant properties. But inspite of its simplicity, this theory has not been popular. It was shown that, in most cases, the rate-limiting step of drug action is diffusion of drugs through distinct barriers, and, in some cases, the generation of response from a stimulus may be rate-limiting (Furchgott, 1964; Roberts and Stephenson, 1976).

1.1.4. Molecular Perturbation Theory

In an attempt to explain the molecular events following drug-receptor interaction which result in a pharmacological effect, Belleau (1964) proposed the molecular perturbation theory. This theory agrees with Koshland's model of enzyme flexibility and substrate-induced fit (Koshland, 1958). It was proposed that agonists induce specific conformational perturbations in the receptor whereas antagonists induce non-specific conformational perturbations, and that the former lead to a biological response while the latter do not. Accordingly, the type of perturbations as well as the rate of these conformational changes were used as parameters to determine extent of agonist action of drug compounds. Although this theory focussed on the important issue of the role of receptor conformations in drug action, it was limited in its scope to a rather general qualitative treatment.
1.1.5. The Two-State Receptor Model and Inverse Agonism

Over the years, it was assumed that in the resting state, receptors existed in an "inactive" state, and were activated only upon ligand binding. Recently, expression of constitutively active G-protein-coupled receptors in cell lines and recombinant receptor systems (Costa and Herz, 1989; Samama et al., 1993; Adie and Milligan, 1994), has raised questions about this assumption. Pharmacological studies in these systems revealed the ability of antagonists to inhibit in varying degrees the constitutive G-protein mediated biochemical activities of these receptors. These antagonists are now termed "inverse agonists". To explain these observations, some investigators have described a two-state model of receptor activation (Leff, 1995; Milligan et al., 1995). In this model, the receptor co-exists in an inactive (R) and an active (R*) state, and it can couple to the G-protein only in the R* state. A ligand may have preferential affinity for the receptor in the R or R* state. If the ligand has higher affinity for the R* state, it will displace the equilibrium of free receptors towards the active state and behave as an agonist, whereas an inverse agonist, due to higher affinity for the R state, will displace the equilibrium towards the inactive state and reduce the extent of G-protein coupling. A true antagonist will have equal affinities for the R and R* states of the receptor, and will not perturb the existing equilibrium. In contrast to existence of the receptors in two states, other researchers have proposed that the receptor is a flexible entity like an allosterically regulated enzyme that catalyzes exchange of GDP for GTP (De Lean et al., 1980; Costa et al., 1992). An agonist would be a positive allosteric modulator, an inverse agonist
a negative modulator, and an antagonist would be neutral, i.e., it will have no effect on the GDP-GTP exchange rate. The essential difference between the two theories is that one assumes the existence of the receptor in two rigid states (R, R*), while the other assumes the receptor to be a flexible protein that can take up a number of different conformations depending upon the type of ligand that is bound, with different resultant affinities for the G-proteins. Dependent upon the type of ligand, the latter theory proposes changes in the affinities of the receptor for the G-proteins rather than in proportion of the receptors distributed between the R and R* states. While the concept of inverse agonism has gained considerable acceptance, the different molecular mechanisms of ligand-receptor interactions are currently being debated and discussed.

1.2 β-Adrenoceptors

1.2.1. History and Classification

The early half of this century witnessed several studies of chemical substances on animal tissues, resulting in the pharmacological classification of neurohumoral systems as parasympathetic or cholinergic, and sympathetic or adrenergic, depending on whether the primary neurotransmitter was acetylcholine or norepinephrine. It is now well known that both of these systems are integral to the physiology and well-being of the body. While both systems exist and function in concert, the cholinergic system is dominant under normal conditions whereas the adrenergic system takes over under
circumstances of stress. The neurotransmitters and hormones belonging to these systems act via receptors that have distinct structures and mediate different functions.

Upon observing differences in the abilities of epinephrine, norepinephrine and related agonists to regulate various physiological processes, Ahlquist (1948) classified the adrenergic receptors (adrenoceptors, ARs) on smooth muscles into α and β, based on excitatory and inhibitory responses of catecholamines, respectively. The catecholamine-mediated vasoconstriction, uterine and nictitating membrane stimulation were attributed to α-AR mediated activities whereas vasodilation and relaxant responses on the uterus, gut and other smooth muscles, as well as stimulation of the heart were considered to be β-AR mediated. This initial classification was corroborated by subsequent studies with antagonists which selectively blocked the α-ARs e.g. ergot alkaloids and haloalkylamines, or the β-ARs e.g. dichloroisoproterenol (Dale, 1906; Nickerson, 1949; Powell and Slater, 1958).

Lands et al. (1967a) further classified the β-adrenoceptors (β-AR) into β₁- and β₂-AR based on rank order of agonist potencies of catecholamines and related analogs in different tissues. The β-ARs in heart and adipocytes were classified as β₁-subtype (isoproterenol > epinephrine = norepinephrine) whereas those in the trachea and blood vessels were termed as β₂-AR (isoproterenol > epinephrine > norepinephrine). Additionally, the β-ARs in small intestine were designated as β₁-AR while those in uterus and diaphragm were β₂-ARs (Lands et al. 1967b). However, the designation of lipolysis-mediating β-ARs in adipocytes as β₁-subtype was questioned when unusually...
low antagonist potencies for propranolol were observed for blockade of norepinephrine-induced lipolysis (Fassina, 1967) and the $\beta_1$-AR selective antagonist, practolol, was able to block isoproterenol-mediated increase in heart rate, but not lipolysis (Stanton, 1972). Also, the isomeric activity ratios for antagonist enantiomers in rat adipocytes were found to be lower compared to the values in rat atria (mainly $\beta_1$-AR) and diaphragm ($\beta_2$-AR) (Harms et al., 1977). Differences in isomeric activity ratios for enantiomers between tissues indicates differences in the receptors mediating responses in those tissues (Patil et al., 1974).

Although several explanations including presence of hybrid receptor with dual characteristics of $\beta_1$- and $\beta_2$-subtypes were proposed (De Vente et al., 1980) the nature of $\beta$-ARs in adipocytes remained controversial until a group at Beecham Research Laboratory, UK, published studies of a novel class of phenethylamines (BRL analogs) on the selective thermogenesis and lipolysis in brown and white adipocytes, respectively (Arch et al., 1984; Wilson et al., 1984). The rank orders of potencies of these compounds for thermogenesis and lipolysis in rodents were different from those for $\beta_1$-AR mediated activities in atria and $\beta_2$-AR mediated activities in uterus and trachea. Moreover, the $pA_2$ values of selective and non-selective $\beta_1$-$\beta_2$-AR antagonists were much lower on adipocytes than on atria and trachea (Wilson et al., 1984). BRL 37344 was found to be more potent than isoproterenol for lipolysis in rat brown adipocytes (Arch et al., 1984). These important differences in pharmacological properties led to the classification of this receptor as "atypical $\beta$-AR". Soon, other independent groups supported the existence of the
atypical β-AR in adipocytes (Bojanic et al., 1985; Hollenga and Zaagsma, 1989; Granneman and Lahners, 1992). Subsequently, atypical β-ARs were also identified in several gastrointestinal tissues such as distal colon (Bianchetti and Manara, 1990), ileum (Blue et al., 1989), esophagus (De Boer et al., 1995; Lezama et al., 1996) and stomach fundus (Cohen et al., 1995). The atypical β-AR was also found to co-exist with other subtypes in the heart (Kaumann, 1989) and skeletal muscle (Sillence et al., 1993). Currently, the atypical β-AR in adipocytes is best characterized and termed as the β3-AR (Strosberg and Pietri-Rouxel, 1996). Atypical β-adrenergic receptors found in other tissues are also included under this subtype despite minor differences in their pharmacological properties.

Earlier studies of β-AR receptor characterization required tedious isolation and purification procedures from native tissues. In the mid 80's, development of molecular biology and recombinant DNA technologies revolutionized scientific research. Using probes derived from the cDNA of purified hamster lung β2-AR, β2-ARs from mouse, rat and human sources were cloned (Dixon et al., 1986). Molecular cloning and expression of other β-AR subtypes from various species too were later successfully carried out in bacterial and mammalian host systems (Marullo et al., 1988; Tate et al., 1991; Muzzin et al., 1991; Granneman et al., 1992; Frielle et al., 1987). Recombinant expression of the β-AR subtypes retained the properties observed in native tissues and provided convenient, low cost homogeneous systems for further characterization of the structure and function of these receptors. Mammalian host systems, such as Chinese hamster ovary cells
(CHO cells), offered the added advantage of coupling to transducing proteins and catalytic units required for signal transduction and biochemical response (Tate et al., 1991).

1.2.2. β-AR-Mediated Signal Transduction

The β-ARs belong to the superfamily of seven-transmembrane spanning receptor proteins that couple to guanine nucleotide-binding proteins or G-proteins (Lefkowitz et al., 1983; Kenakin, 1996). Agonist binding to the receptor induces conformational changes leading to receptor activation which facilitates coupling to the G-protein. G-proteins are membrane-bound heterotrimeric proteins composed of three distinct subunits: α subunit of 39-46 kDa, β subunit of 37 kDa and γ subunit of 8 kDa (Gilman 1987; Hepler and Gilman, 1992). The β and γ subunits exist as a tightly associated complex and function as a unit. Although several different isoforms of each of these subunits are now discovered with resultant variations in heterotrimeric combinations and thereby different regulatory implications (Sternweis, 1994; Taussig et al., 1994; Neer, 1995), the G-proteins are primarily classified based on the type of α subunit present. In the activated state, the β-ARs are known to couple with Gαi-proteins which consist of the αi-subunit. Coupling of the receptor converts the inactive, GDP-bound αi subunit to its active state which binds GTP in the presence of Mg++2. This promotes the dissociation of the G-αi subunit from the stationary β γ dimer and migration along the cytoplasmic wall of the cell membrane to activate the membrane bound enzyme, adenylyl cyclase (Northup et al., 1980). In addition, Gαi can also enhance the
activation of voltage-sensitive Ca\(^{2+}\) channels in the plasma membrane of skeletal and cardiac muscle (Brown and Birnbaumer, 1988). The \(\alpha\) subunit also possesses intrinsic GTPase activity, which hydrolyzes the bound GTP to GDP and P\(_i\). Removal of the GTP terminates the active state of the protein, and the GDP-bound form reassociates with the \(\beta\,\gamma\) subunits until the next cycle of \(\beta\)-AR coupled reactivation (Lee et al., 1992; Hepler and Gilman, 1992).

Adenylyl cyclase is a membrane bound enzyme of molecular mass around 120 kDa, consisting of a single polypeptide comprising a cytoplasmic N-terminus and two more or less identical domains, each consisting of six transmembrane spanning helices followed by long cytoplasmic loops, ending in a cytoplasmic C-terminus (Krupinski et al., 1989). When activated, this enzyme catalyzes the conversion of ATP to cAMP and pyrophosphate in the presence of Mg\(^{2+}\) (Birnbaumer et al., 1969). Exogenous compounds such as forskolin activate adenylyl cyclase by mimicking G\(\alpha\), activity. At least eight different isoforms of this enzyme are now known to exist, (Krupinski et al., 1992), and each isoform is regulated differently by the various \(\alpha\) and \(\beta\,\gamma\) subunits of G-proteins, and/or Ca\(^{2+}\)/calmodulin present in the cells (Ueda and Tang, 1993; Taussig et al., 1994). It has been suggested that \(\beta\)-AR agonists increase the expression of only type III adenylyl cyclase (Granneman, 1995).

With the ongoing discovery of new isoforms of G-proteins and adenylyl cyclases and of their roles in functional and regulatory mechanisms, the agonist-activated, \(\beta\)-AR mediated increase in cAMP in cells is now believed to be not just a simple amplification process but rather the net result of several
complex and multidirectional regulatory processes generated within the cell machinery. Yet, the agonist-induced increases in cAMP levels yield a fairly convenient and specific, measurable parameter to evaluate β-AR activation. In fact, the correlation of increase in intracellular levels of cAMP to β-AR activation was established much earlier by Sutherland and Rall in 1957. It is well known now that cAMP acts as an important second messenger in cellular signaling pathways. Increased levels of cAMP are known to activate cAMP-dependent protein kinase (PKA) which then phosphorylates various cellular proteins and thereby propagates mechanisms resulting in biochemical and functional response (Taylor et al., 1992; Francis and Corbin, 1994). Figure 1 depicts the pathway of β-AR-mediated signal transduction that leads to different biochemical responses within cells, depending upon tissue type.

1.2.3. Structure of the β-AR

The β-AR is a cell membrane-bound singular polypeptide, and depending upon the subtype and species, it is comprised of about 400-470 amino acids with molecular mass of 62-67 kDa. The secondary and tertiary structure of the β-AR is similar to those of other G-protein coupled receptors including α-AR, serotonin and muscarinic (m2) receptors. Electron diffraction and hydropathy plots revealed seven regions of hydrophobic amino acids (20-30 amino acids each) forming the characteristic transmembrane α-helical domains which are linked by extracellular and intracellular loops of hydrophilic residues, similar to the earlier identified topology of bacteriorhodopsin and mammalian rhodopsin (Henderson and Unwin, 1975; Dixon et al., 1986; Lefkowitz and
Caron, 1988). The amino and carboxyl termini of the receptor are located in the extracellular and cytoplasmic regions, respectively (Dixon et al., 1986; Dohlman et al., 1987). So far, β2-AR has been the most extensively studied receptor, but more recently, the primary structures of all the three β-AR subtypes obtained from different species including human, mouse and rat, have been deduced and compared. Structural homologies were found to be as high as 80-90% between same subtypes in different species. Homology is less for different subtypes in the same species, ranging between 40-50% (Strosberg, 1995). Figure 2 illustrates the primary structure of the human β3-adrenoceptor (Strosberg and Pietri-Rouzel, 1996).

1.2.3.1 Extracellular Domains of the β-AR

Two N-glycosylation sites have been identified on the N-terminus of the receptor, corresponding to positions 6 and 15 of β2-AR (Dohlman et al., 1987; Benovic et al., 1987). Enzymatic removal of the carbohydrates did not affect ligand binding or the G-protein coupling. Lack of polysaccharide also did not affect functional expression of β-ARs in E. coli (Strosberg and Marullo, 1992). However, absence of carbohydrates does appear to reduce the density of β2-AR expressed on A431 cells (Cervantes-Olivier et al., 1988), and absence of N-glycosylation in mutant β-AR in L cells decreased the agonist affinity and receptor-mediated activity (Rands et al., 1990). N-Glycosylation may thus be essential for cellular anchoring and function of these receptors in some cells.

Disulfide bonds are also considered to play an important role in β-AR function. Two such bonds have been identified in the second extracellular
loop of the \( \beta \)-AR, one formed between Cys\(^{106} \) and Cys\(^{184} \), and the other between Cys\(^{190} \) and Cys\(^{191} \) in the \( \beta_2 \)-AR. These bonds are found in homologous positions on the other two subtypes too. Based on results from site-directed mutagenesis and functional studies, at least the former bond has been suggested to be essential for ligand binding to the receptors (Dixon et al., 1987a; Fraser, 1989; Dohlman et al., 1990).

1.2.3.2. Transmembrane Domains of the \( \beta \)-AR

The seven membrane-spanning hydrophobic domains are the most conserved regions of the \( \beta \)-ARs and form the ligand-binding pocket. All the seven transmembrane (tm) regions are essential, and deletion of any of these domains abrogates ligand-receptor interactions (Dixon et al., 1987a,b). On the other hand, removal of most of the amino- or carboxy-terminal portions by proteolysis (Rubenstein et al., 1987; Wong et al., 1988) or by deletion mutations of \( \beta_2 \)-AR (Dixon et al., 1987a) or \( \beta_3 \)-AR (Strosberg, 1995) has little or no effect on the binding of ligands to the receptors. Additional support for this theory has been provided by the localization of retinal 22 Å within the membrane bilayer of rhodopsin (Thomas and Stryer, 1982) and by fluorescence quenching derived localization of the antagonist, carazolol, at least 10.9 Å deep into the hydrophobic core of the \( \beta_2 \)-AR (Tota and Strader, 1990). Construction of \( \alpha_2/\beta_2 \)- and \( \beta_1/\beta_3 \)-AR chimeras with systematic exchange of receptor domains, resulted in altered ligand binding specificity from one subtype to the other (Kobilka et al., 1988; Frielle et al., 1988). Structure-activity relationship studies of \( \beta_1 \)- and \( \beta_2 \)-AR ligands (Main and
Tucker, 1985) indicated that β-agonists are catecholamine mimics containing a 3,4-substituted catechol ring connected to a protonated amine group via a β-hydroxyl ethyl side chain. Substituents on the amine influenced the receptor affinity and subtype specificity, while the catechol ring was essential for agonist activation of the receptor. In contrast, antagonists are characterized by the presence of a more hydrophobic group, often consisting of a fused ring system, connected to the amine by a β-hydroxypropyl ether side-chain. Based on this knowledge and genetic analysis of the β-AR, site-directed mutagenesis of the receptors was carried out (Strader et al., 1987a,b). Substitution of Asp$^{113}$ in tm3 with Glü$^{13}$ decreased the affinity by 10-fold, whereas substitution with Asn$^{13}$ resulted in a 10,000-fold decrease in binding affinities of ligands, indicating that ionic interaction between the carboxyl side chain of Asp$^{113}$ and the protonated amine of the ligand is more or less essential for ligand binding to the receptor (Strader et al., 1988). Substitution of the aspartate with Ser$^{113}$ resulted in activation of the mutant receptors by catechol esters and catechol ketones which failed to activate the wild type receptors (Strader et al., 1991). These data suggest that although the amine moiety is a major contributor to the overall binding energy of interaction, the position of catechol ring is critical for receptor activation.

Site-directed mutagenesis and structure activity relationship studies also led to the identification of Ser$^{204}$ and Ser$^{207}$ in tm5 as residues critical for ligand binding to the β$_2$-AR. Substitution of either residue with Ala resulted in 10-fold decrease in agonist binding and 50% reduction in the agonist efficacy, without affecting antagonist interactions (Strader et al., 1989).
Similar results were obtained with wild type receptors when either hydroxyl group of the catechol of the ligand was absent. Detailed studies indicated hydrogen-bond interactions of Ser$^{204}$ and Ser$^{207}$ residues with the meta- and para-hydroxyl groups of the catechol, respectively, while Phe$^{290}$ in tm6 of the receptor interacts with the aromatic catechol ring of the ligand, and orients the ligand specifically in the binding pocket (Dixon et al., 1988). As for other residues identified to be involved in specific interactions, based on molecular modeling, the chiral β-hydroxyl group of the ligands is postulated to interact with a serine residue in tm4 or tyrosine residue in tm7 (Lybrand, 1996). Also, substitution of the highly conserved Asp$^{79}$ and Asp$^{130}$ in tm2 and tm3, respectively, of the β$_2$-AR, selectively reduced agonist binding and G-protein coupling, and did not affect antagonist binding, suggesting a role for these residues in agonist-induced conformational changes (Chung et al., 1988; Fraser et al., 1988; Strader et al., 1988). More recently, binding studies with chimeric β$_2$/β$_3$-ARs suggested that the tm5 region of the β$_3$-AR may contain critical determinants for the high affinity of BRL 37344 for the receptor (Guan et al., 1995).

1.2.3.3. Cytoplasmic Domains of the β-AR

Deletion mutation, site-directed mutagenesis and chimeric receptor studies have provided strong evidence for the role of cytoplasmic loops and the carboxy terminus of the β-AR in G-protein coupling and receptor regulation. While the presence of all the three intracellular loops is essential, conservation of residues in the C-terminal region of the second loop, the N-
and C-terminal portions of the third intracellular loop and the N-terminal portion of the carboxy terminus is critical for G-protein coupling and activation of adenylyl cyclase (Dixon et al., 1987a, 1988; O’Dowd et al., 1988; Strader et al., 1987a). Studies with mutant β3-ARs lacking small segments of N- or C-terminal domains of the third intracellular loop indicated this segment to be important for coupling to G-proteins and suggested a role for these regions in maintaining the resting state of the unliganded β3-AR (Guan et al., 1995). In the β2-AR, palmitoylation of Cys341 has been shown to contribute significantly to the ability of the agonist-bound receptor to mediate adenylyl cyclase stimulation, possibly by forming a fourth intracytoplasmic loop resulting in an active conformation for G-protein coupling (O’Dowd et al., 1989; Guan et al., 1995). Lack of palmitoylation resulted in increased phosphorylation of the receptor, suggesting that the absence of the fourth loop increases exposure of phosphorylation sites on the receptor (Moffett et al., 1993). Studies with mastoparan, a bee venom peptide that forms an amphiphilic α-helix in solution and stimulates G-proteins (Higashijima et al., 1990), indicate that similar amphipathic α-helices are formed by residues at N- and C-termini of the third intracellular loop, contributing to G-protein activation. However, the second intracellular loop is also involved in the G-protein coupling (Liggett, 1991; Cotecchia et al., 1991).

In addition to G-protein coupling, the intracellular domains of β-ARs are also implicated in homologous and heterologous desensitization and down-regulation of the receptor upon prolonged agonist exposure. Agonist-induced activation of β-adrenergic receptor kinase (β-ARK) and PKA results in
phosphorylation of specific residues on the β-AR. Consensus sequences of PKA-mediated phosphorylation have been identified on the third intracellular loop of the β₁- and β₂-ARs (Liggett, 1991). Phosphorylation of these sites renders the receptor incapable of coupling to G-proteins (Pippig et al., 1993; Freedman et al., 1995; Post et al., 1996). On the other hand, several serine and threonine residues on the cytoplasmic tail of the β₁- and β₂-ARs are phosphorylation sites for β-ARK, which results in subsequent binding of the protein ß-arrestin to the receptor and thereby, uncoupling of the agonist-occupied receptor from the G-proteins (Benovic et al., 1986; Lohse, 1993). The β₃-AR seems to lack the residues that constitute the phosphorylation sites for PKA and β-ARK as it does not undergo short-term desensitization in most cell systems. Insertion of some of these sequences in chimeric β₂/β₃-ARs resulted in partial restoration of desensitization (Nantel et al., 1993). However, other sequences present in all the three β-AR subtypes could be phosphorylated by different kinases and thus contribute to heterologous desensitization of the β-ARs (Jockers et al., 1996).

1.3 Affinity and Photoaffinity Binding

1.3.1 Chemistry and Application

Compounds which interact irreversibly with proteins serve as important pharmacological and biochemical tools with wide potential applications. These compounds have been traditionally used for identification and characterization of receptors, enzymes as well as regulatory and immunological proteins, and
are categorized as (chemical) affinity or photoaffinity ligands. Affinity labeling of proteins involves two distinct steps: selective binding to the protein of interest and irreversible attachment to the protein, possibly through covalent bond formation. This is achieved by incorporation of a chemically labile group into a ligand of high affinity with retention of structural features required for macromolecular interaction. The modified ligand can become an affinity label when a radioactive, fluorescent or spin-labeled marker is incorporated for detection purposes (Sokolovsky, 1984).

Chemical affinity ligands or alkylating ligands possess electrophilic groups, which attack nucleophilic (electron-rich) nitrogen, oxygen or sulfur atom of an amino acid to form a covalent bond. Some examples of chemically reactive groups that participate in covalent interactions with proteins include: haloacetyl (i.e. XCH2CO where X = Cl, Br, or I), sulfonylhalides (SO2F), halo-ketones, isothiocyanato (-NCS), epoxides, azaridines and diazo derivatives (Sokolovsky, 1984). The nucleophilicity of an amino acid and the reactivity of a reagent group determine the overall rate of affinity binding. Some of the amino acids with nucleophilic side chains include cysteine, methionine, histidine, lysine, tryptophan, arginine, aspartate, glutamate and tyrosine (Sweet and Murdock, 1987). The affinity label may attack an amino acid residue within the ligand binding site, or within the vicinity of the site such that the covalently linked ligand blocks access of substrates to the binding site. In general, design of compounds as affinity ligands is required to fulfil the following criteria: (1) they must possess sufficient affinity for binding to the receptor of interest, (2) the reagent group must be stable throughout the
affinity alkylation process, (3) the reagent group should be sufficiently reactive to rapidly form a covalent bond with amino acids of the protein, and (4) the modified amino acid should be readily identifiable. Non-specific labeling of proteins is a commonly encountered problem with affinity labels possessing high reactivity, however, it can be overcome by differential labeling of proteins in the absence and presence of a specific protective agent such as an inhibitor, agonist or antagonist (Sokolovsky, 1984).

The photoaffinity technique is a variant of the affinity labeling by alkylation agents. In this method, the photoaffinity ligand can bind specifically and reversibly with the binding site of the protein, and contains a photolyzable group which is unreactive in the dark, but converts to an extremely reactive intermediate when exposed to light of appropriate wavelength. The reactive intermediate may then form a covalent bond with residues in the binding site of the receptor before the ligand dissociates from the site (Sokolovsky, 1984). The photolyzed intermediates of most successfully utilized photoaffinity agents are relatively non-selective and of higher reactivity than electrophilic groups of chemical affinity agents. However, prior reversible occupation of the binding site by these compounds offers potential advantage of maximizing the specificity of covalent attachment at the binding site upon exposure to light. Although the degree of specificity of covalent binding by both, alkylation agents and photoaffinity reagents, is directly proportional to their binding affinities for the receptor, relatively greater specificity of labeling is achieved with photoaffinity analogs of ligands that possess low binding affinities (Guillory, 1989). The photolyzable groups commonly employed are
diazooacetyl and arylazides, which upon irradiation are converted to carbene and nitrene radicals, respectively (Sokolovsky, 1984). Arylazides are the preferred over diazoacetyl derivatives for photolabels, since carbenes exhibit a high degree of non-specificity. Nitrenes are less indiscriminate in their reactions with the proteins and are also somewhat electrophilic, preferring an O-H over a C-H bond. The lifetimes of these intermediates are extremely short, i.e. nanoseconds to milliseconds (Ruoho et al., 1994). Therefore, even ligands with low dissociation constants between $10^{-6}$ and $10^{-4}$ M can react before dissociation can occur from the binding site. A major drawback of arylazides for use as nitrene precursors though, is their susceptibility to reduction to corresponding amines, especially in a reducing environment (Guillory, 1989). Damage to proteins upon exposure to UV light is another potential problem with photoaffinity labeling. However, the very high extinction and quantum yields of many arylazides at 250-280 nm often allows rapid photolysis within a time scale precluding biological damage (Guillory, 1989).

1.3.2. Affinity and Photoaffinity Labeling of the β-AR

One of the first compounds shown to bind irreversibly to receptors was dibenamine, which alkylates α-ARs, muscarinic, 5-HT and histamine receptors (Harvey and Nickerson, 1953; Furchgott, 1954). Dibenamine and phenoxybenzamine were used for receptor inactivation in studies involving determination of agonist dissociation constants (Furchgott, 1966; Furchgott and Bursztyn, 1967). However, despite the structural similarity with these members of the family of seven transmembrane-bearing G-protein coupled
receptors, irreversible blockade of the β-ARs by haloalkylamine derivatives was less successful. In 1976, Takayanagi et al. reported photoaffinity labeling of β-ARs from guinea pig caecum using l-isoproterenol and 2-(2-hydroxy-3-isopropylaminoproxy)iodobenzene derivatives, while in another study, N-bromoacetylethylene diamine derivative was used for irreversible blockade of β-ARs in cardiac tissue (Venter, 1979). The applications of these affinity and photoaffinity agents, though, were directed towards irreversible blockade of the fraction of spare β-AR receptors. One of the earlier uses of photoaffinity labels as tools for characterization of the β-AR was successfully demonstrated by Lavin et al. in 1981, in which [³H]-p-azidobenzylcarazolol was shown to bind to a 58 kDa peptide of the β₂-ARs in frog erythrocyte membranes. [¹²⁵I]-Radiolabeled derivative of the same compound was later prepared and utilized for characterization of β₁- and β₂-ARs in various animal tissues (Lavin et al., 1982; Heald et al., 1983; Nambi et al., 1984). Simultaneous breakthroughs in the development of affinity chromatography systems for purification of the receptors along with the use of the photoaffinity agent facilitated elegant studies on the β-ARs to probe the molecular properties of the receptor and gain insights into mechanisms of receptor desensitization (Lefkowitz et al., 1984, Benovic et al., 1984). The alkylating derivative, p-(bromoacetamido)-benzylcarazolol was also later synthesized and used in similar studies of receptor characterization (Dickinson et al., 1985; Dohlman et al., 1988). Some reports using other photoaffinity and affinity labels in studies with β-ARs include the azido derivative of iodocyanopindolol (Bergermeister et al., 1982), and the bromoacetyl
derivatives of pindolol (Molenaar et al., 1988) and cyanopindolol (Bar-Sinai et al., 1986), respectively. Synthesis of novel photoaffinity derivatives of prenalterol and tyramine for the \( \beta \)-AR has also been reported (Ruoho et al., 1994). More recently, antagonist interactions with the \( \beta_2 \)-AR were characterized using photoaffinity analogs of antagonists that carried the photoactivable group either on the aryloxy end or the amino end of the molecule (Hockerman et al., 1996). Thus, along with the development of molecular biology techniques, affinity and photoaffinity labeling studies with these compounds has contributed significantly to our current knowledge of \( \beta \)-ARs in recent years.

1.4 \( \beta \)-AR Activity of Trimetoquinol (TMQ)

1.4.1. History of TMQ

Trimetoquinol (TMQ) is chemically described as 1-(3'4'5'-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (see figures 3, 4 or 6 for TMQ structure). The S(-)-isomer of TMQ is currently used in Japan as a bronchodilatory agent. The structure of TMQ is based on that of tetrahydropapaveroline, a demethylated and reduced analog of papaverine, which was earlier shown to induce increase in heart rate, decrease in blood pressure, bronchodilation and relaxation of uterine smooth muscle in animals (Laidlaw, 1910). These effects were later established to be \( \beta \)-AR mediated (Holtz, 1966). The potent bronchodilatory activities of TMQ among a series of tetrahydroisoquinoline analogs were initially reported by Iwasawa and
Kiyomoto in 1967. TMQ was shown to be 10-fold more potent than isoproterenol for producing bronchodilatory effects. In other studies TMQ was also shown to mediate hypotensive and positive cardiovascular effects that were blocked by pronethalol, a β-AR antagonist (Sato et al., 1967). Subsequently, it was established that the levorotatory S(-)-isomer of TMQ mediated the β-AR effects (Kiyomoto et al., 1969) whereas the dextrorotatory R(+)isomer was a potent antagonist of thromboxane A₂ receptors (Dalton et al., 1976). In guinea pig aorta, TMQ behaved as a weak α-AR antagonist (Lee et al., 1974). In rat epididymal fat pads and in rat adipose tissue, S(-)TMQ exhibited potent lipolytic activity, which was blocked by β-AR antagonists (Shonk et al., 1971; Lee et al., 1974). Based on the structural similarity of TMQ to papaverine which behaves as a phosphodiesterase inhibitor, the increases in cAMP by TMQ were suggested to occur by the same mechanism (Inamasu et al., 1974), but soon this hypothesis was disproved when it was shown that the β-AR activity of TMQ does not correlate with an inhibition of cAMP phosphodiesterase (Piascik et al., 1976). Moreover, the increase in cAMP in fat cells was blocked by propranolol, suggesting that the lipolytic response was by β-AR mediated activation of adenylyl cyclase (Piascik et al., 1978). It is now well established that the lipolytic response in adipocytes is mediated mainly by the β₃-AR subtype in these cells (Wilson et al., 1984).
1.4.2. Structure Activity Relationship of TMQ

1.4.2.1. Substitutions on the Tetrahydroisoquinoline Backbone

While the β-hydroxyl group of catecholamines was shown to be important for β-AR mediated activities (Feller and Finger, 1970), it was not necessary for TMQ which was a closed ring analog and possessed a bulky aromatic side chain (Shonk et al., 1971). Presence of a 1-methyl substituent (salsolinol) on the catechol-containing tetrahydroisoquinoline nucleus as well as the 3',4'-dihydroxybenzyl substituent (tetrahydropapaveroline) reduced the potency of the compounds at the β-ARs as compared to the 3',4',5'-trimethoxybenzyl group of the parent compound, TMQ (Lee et al., 1974; Feller et al., 1975). The corresponding opened ring analog, N-(3',4',5'-trimethoxyphenethyl)-dopamine was a weak β-AR agonist, indicating that the presence of a bulky lipophilic group and the fixed stereochemistry of TMQ compensate for the absence of the β-hydroxyl group for interactions with the β-ARs (Miller et al., 1975). Addition of hydroxy or methyl groups at the 1-position of TMQ gave rise to erythro and threo isomers of analogs that were less potent than TMQ with the exception of threo-α-methylTMQ which exhibited greater bronchodilatory potency than the parent compound, and thereby, higher β₂/β₁-AR selectivity (Piascik et al., 1978; Mukhopadhyay et al., 1982). Substitutions at the amino nitrogen of TMQ gave rise to tertiary amine analogs and resulted in decreased potencies in guinea pig atria and trachea (Adejare et al., 1986; Fedyna et al., 1987; Shams et al., 1990a). The decrease in potencies of these analogs correlated with increasing bulk of the substituents,
indicating steric hindrance for interaction of the protonated amine with the β-ARs. In studies with Asp113Asn mutated hamster β2-ARs, the important contribution of ionic interaction of between the secondary amine of TMQ with Asp113 on the receptor was demonstrated by the fact that agonist activities of TMQ were reduced in the mutated receptors compared to the wild type (Fraundorfer, 1993). However, the TMQ analogs were more potent than (-)-isoproterenol in the mutated receptor system. Halogen substitutions at the 5- and 8-positions of the catechol ring of TMQ increased β2-/β1-AR selectivities, but decreased functional potencies relative to TMQ (Clark et al., 1987; Miller et al., 1988; Markovich et al., 1992) although halogen substitutions at 5-position enhanced affinities for the β-ARs as opposed to those at 8-position (Fraundorfer et al., 1994a). Thus, electronic factors as well as steric bulk at these positions seem to influence the activities of TMQ analogs on the β-ARs (Markovich et al., 1992; Fraundorfer et al., 1994a).

More recently, the significance of interactions of TMQ with serine residues in tm5 of hamster β2-AR was demonstrated by studies with mutated receptors (Ser204Ala, Ser207Ala) that resulted in decreased agonist activities of TMQ and its analogs in this system (Fraundorfer, 1993). The Ser207Ala mutation was found to affect the biochemical potencies to a greater extent than Ser204Ala. It is postulated that similar to the interactions of catecholamines, the mutations abolished interactions of the catechol hydroxyl groups of TMQ with the serine residues, which may be contributing significantly towards agonist-induced conformational changes in the receptor. Thus, from these studies, it can be inferred that the tetrahydroisoquinoline
portion of TMQ interacts with the β-ARs at a ligand-binding site common to other catecholamine β-AR agonists.

1.4.2.2. Substitutions on the 1-Benzyl Side-chain of TMQ

As mentioned earlier, the 1-(3',4',5'-trimethoxy)-benzyl side-chain is important for the agonist activities of TMQ on β₁- and β₂-AR systems as well as for lipolysis (Feller et al., 1975). To further characterize the significance of this side-chain, functional activities of several 1-benzyl substituted analogs have been evaluated. When selected compounds with substitutions at the 3'- and/or 5'-positions of the benzyl group were tested on guinea pig atria (β₁-AR) and trachea (β₂-AR), these had reduced agonist activities and potencies on both systems, except for the 3'-nitro substituted analog which showed higher potency compared to TMQ in the atria (Shams et al., 1990a). Since these activities were blocked by propranolol in a competitive manner, it was confirmed that the compounds interacted with β-ARs in the tissues. The 3'-amino and 3'-demethoxy analogs showed only marginally (2-3-fold) higher β₂/β₁-AR selectivity compared to TMQ, whereas in contrast, the 3'-nitro analog had 8-fold higher selectivity for β₁- over β₂-AR. Interestingly, substitution of 3'- and/or 5'-methoxy groups with iodine atoms markedly reduced β₁- and β₂-AR mediated agonist activities in the guinea pig tissues (Shams et al., 1990b) although the binding affinities of these compounds were higher for these β-AR subtypes as compared to the parent compound (Fraundorfer et al., 1994a). Biochemical studies in CHO cells expressing the hu-β₂-ARs indicated that these high affinity iodinated analogs did not stimulate
adenylyl cyclase to the same extent as (-)-isoproterenol or S(-)TMQ. On the other hand, these analogs demonstrated higher activities in biochemical studies with L cells expressing Asp113Asn mutated hamster β2-ARs. These studies indicate that compared to the trimethoxybenzyl group, the iodine-substituted side-chain of TMQ is able to stabilize the receptor interactions to a greater extent, but the ability to induce conformational changes in the β2-AR for agonist activity is reduced (Fraundorfer, 1993). More importantly, in functional studies on rat atria and trachea, the 3',5'-diiodo analog of TMQ was a partial agonist with potencies significantly lower than TMQ. In contrast, this analog was a full and potent agonist for relaxation of rat distal colon and lipolysis in rat brown adipose tissue (Konkar et al., 1996). Also, in CHO cells expressing the rat β3-AR, the diiodo analog exhibited higher affinity and potency for cAMP accumulation compared to TMQ and (-)-isoproterenol, and these values closely paralleled those reported earlier for the selective β3-AR agonist, BRL 37344 (Muzzin et al., 1991). These results were in agreement with other investigations which indicated that β-AR ligands with large substituents on the amino nitrogen exhibit antagonist or partial agonist activities at β1- and β2-ARs but are potent agonists at the atypical-β/β3-ARs (Mohell and Dicker, 1989; Sugasawa et al., 1992; Blin et al., 1993). Collectively, the above studies suggest that the trimethoxybenzyl side-chain of TMQ contributes to the affinity and agonist activity of the compound on β-ARs, but this side-chain plays a more important role in agonist activity at the β3-ARs, than at β1- or β2-ARs.
1.5. Statement of the Problem

1.5.1. Rationale and Objectives

Selectivity of agonist activities of several compounds including TMQ, at the $\beta_3$-AR (vs. $\beta_1$- and $\beta_2$-ARs) or the lack of it, may possibly be explained by some important similarities and differences in the structures of these receptors, most importantly, the seven transmembrane domains of these receptors which form the ligand binding pocket. Several studies in the recent years involving site-directed mutagenesis (Strader et al., 1987a,b; Dixon et al., 1988; Dixon et al., 1989; Strader et al., 1989), chimeric receptor construction (Frielle et al., 1988; Marullo et al., 1990), fluorescence binding probe analysis (Tota and Strader, 1990) and computer aided three-dimensional modelling (Lewell, 1992; Maloney, Huss and Lybrand, 1992) have helped identify $\beta$-AR sites important for ligand binding and G-protein activation: (1) The most important for binding specificity is Asp$^{113}$ in tm3, which acts as a counter-ion for the charged amino group of the ligand, and is conserved in all adrenergic receptors. (2) Also conserved in all three subtypes are Ser$^{204}$ and Ser$^{207}$ of tm5, which form hydrogen bonds with the meta- and para-hydroxyl groups of catechol possessing agonists while the aromatic ring of the catechol moiety is stacked between Phe and Trp residues of tm5 and tm6. The catechol ring is considered to be essential for agonist activation of the receptor. (3) Hydrogen bond between the $\beta$-hydroxyl group and Ser in tm4 or Tyr in tm7 confers extra stabilization and higher binding affinity of the active stereoisomers of the catecholaminergic agonists.
While most $\beta_1/\beta_2$-AR agonists possess a common backbone of an aromatic ring containing catechol and a secondary charged amine with or without the $\beta$-hydroxyl group, the substituents at the amine consist of short alkyl groups (Blin et al., 1993; Strosberg and Peitri-Rouxel, 1996), conferring a rather compact structure to the ligand which interacts with the receptors predominantly at sites on $\text{tm} 3$, $4$, $5$ and $6$ (Dixon et al., 1988). Figure 3 illustrates the chemical structures of several $\beta_1/\beta_2$-AR agonists, including TMQ. On the other hand, common structural features of most $\beta_3$-AR selective or potent agonists such as BRL 37344, LY 79771, bucindolol, and CL 316,243 as well as salmeterol, a $\beta_2/\beta_3$-AR agonist, (figure 4) are the following: (1) an aromatic ring (substituted or not) lacking the catechol function, (2) (oxy)hydroxylalkylamine with (3) phenyl or indol function carrying hydroxyl, ether or acid functions which increase steric bulk and possess moderate lipophilicity (Blin et al., 1993; Howe, 1993; Arch et al., 1984). This bulky and lipophilic side chain has been postulated to interact with amino acid side chains in $\text{tm} 7$, $1$ and $2$. In fact, three dimensional view of the $\beta_2$- and $\beta_3$-AR sites showing docking of $\beta_2$- or $\beta_3$-AR selective ligands confirm this difference in steric space occupation of the site (Strosberg et al., 1993; Strosberg and Pietri-Rouxel, 1996). It is also suggested that Asp$^{79}$ in $\text{tm} 2$ and Asn$^{319}$ in $\text{tm} 7$ may be selectively involved in agonist binding and signal transduction (Strader et al., 1987a,b). Analysis of amino acids in the $\beta_1$- and $\beta_2$-ARs with those in $\beta_3$-AR show differences which could have important implications on the ligand selectivity: Substitution of Ala (B$\beta_1$-AR) or Phe (B$\beta_2$-AR) by Gly (B$\beta_3$-AR) in $\text{tm} 1$ and replacement of Phe and Phe (B$\beta_1$-AR) or Leu and
Leu (β₂-AR) with Ala and Leu (β₃-AR) in tm7. Replacement of these bulky side-chains of β₁- and β₂-AR renders this site more accessible to ligand molecules. Further, the presence of two additional proline residues in tm7 of β₃-AR may play a direct role in message triggering, permitting conformational changes involved in signal transduction (Blin et al., 1993). Thus, there seems to be strong evidence favoring the hypothesis of an additional site on the β₃-AR that may interact with the relatively lipophilic and bulky side-chain of selective ligands and induce agonist responses at this receptor. Figure 5 depicts a model of the β₃-adrenoceptor ligand-binding region that proposes interactions based on studies mentioned above. The catecholamine ligand, norepinephrine is shown to interact with amino acids in tm3, 4, 5 and 6. The amine group of the ligand is oriented such that a bulky side-chain, if present, would be surrounded by the region composed of tm1, 2, and 7. It would be of great significance to elucidate the details of this site to enable design of ligands with substituents that can result in optimized interaction with the β₃-AR, in order to develop highly potent and selective β₃-AR agonists for desired therapeutic applications.

S(-)TMQ has potent agonist activities on β-ARs and it shares overall structural similarity with several non-selective and selective β-AR agonists (see figures 3 and 4). The catecholamine portion of the molecule shares the following features common to non-selective agonists like norepinephrine and isoproterenol: a catechol-bearing aromatic ring and charged secondary amine with benzylic side chain. While the catechol moiety was essential for agonist activity at the β₁- and β₂-AR, the trimethoxybenzyl side chain of TMQ seems
to provide a feature more common to the $\beta_3$-AR-selective agonists: the presence of a bulky, aromatic side chain on the secondary amine. In line with Blin's hypothesis discussed above, 3',5'-diiodo substitution on this benzyl side chain has demonstrated improved selectivity for the $\beta_3$-AR over the other two subtypes (Konkar et al., 1996). More recently, the combination of masking the catechol group, as in the presence of a bioisoteric aminothiazole ring, coupled with the presence of the 3',5'-diiodo-4'-methoxy substitutions on the 1-benzyl ring produced compounds with selective agonist activity on the human $\beta_3$-AR (Konkar et al., 1996). Further substitutions at the 4'-position of the 3',5'-diiodo-TMQ gave a series of derivatives that exhibited higher binding affinities for the $\beta_3$-AR expressed in CHO cells (Shams et al., 1995). Thus, we hypothesize that the benzylic side-chain of TMO interacts with a site common to that of $\beta_3$-AR-selective agonists, which is as yet undefined, but postulated to reside in the region formed by tms 1, 2 and 7 of the receptors.

The long side-chain of salmeterol, an analog of salbutamol with potent $\beta_2$/$\beta_3$-AR agonist activity (figure 4), is suggested to fit into an exo-site domain of the $\beta_2$-AR (Johnson et al., 1993), and one wonders how modifications of the TMO chemical class will agree or disagree with the hypothesis of Blin et al., 1993 and the observed selectivity with salmeterol. Therefore, in order to characterize the differential interactions of the 1-benzyl side-chain of TMO with the additional site on the $\beta$-AR subtypes, 4'-substituted derivatives of 3'-iodo- and 3',5'-diiodo-TMQ were synthesized for use as affinity and photoaffinity ligands.
The aims of this study are to evaluate:

1) the pharmacological properties of a series of 1-benzyl substituted TMQ analogs i.e. to determine the binding affinities and agonist activities on the β-AR subtypes, and

2) the affinity characteristics of the compounds in order to determine their usefulness for receptor labeling and binding-site characterization in future studies.

The compounds selected for these investigations are shown in Figure 6. The haloacetamido and isothiocyanato groups as in chloroacetamido- and bromoacetamido-DITMQ and in isothiocyanatolTMQ, respectively, are known to be reactive alkylating groups. The photoaffinity TMQ analog, azidolTMQ, should also be useful for labeling these receptors, since the azido group is susceptible to photolysis by UV light. In this regard, the p-(bromoacetamidyl) benzyl and p-azidobenzyl derivatives of carazolol, a very potent and selective β₃-AR agonist and β₁/β₂-AR antagonist, have been successfully used in the past to study β₁- and β₂-ARs (Lavin et al., 1982; Dickinson et al., 1985). These 4'-substituted derivatives of 3'-iodo- and 3',5'-diiodoTMQ may also possess very high affinity for the β₃-AR and may be useful for characterizing the hydrophobic domain of the receptor.

1.5.2. Significance

The traditional therapeutic application of TMQ, like most other β-AR agonists, has been for the treatment of bronchopulmonary diseases. The β₂-AR mediated smooth muscle relaxant properties of this compound have been.
utilized for conditions like asthma and chronic obstructive pulmonary disease. As with many other β-AR agonists, cardiostimulatory and tremorigenic side-effects are often observed with initial clinical use of TMQ. In order to minimize these side effects and improve the therapeutic profile of the drug, attempts have been made over the years to design analogs of TMQ with superior potencies and $\beta_2/\beta_1$-AR selectivities. Characterization of functional groups on the drug molecule which are important for agonist interactions with the receptors, has been extremely useful in designing new analogs. Similar attempts on other β-AR agonist molecules have led to development of compounds like salbutamol and terbutaline, which possess high $\beta_2/\beta_1$-selectivities and therefore widespread use for bronchopulmonary applications. Salmeterol, a more recently designed analog of salbutamol with extended duration of action, has also shown activity on the $\beta_3$-receptor.

Although found to co-exist in most tissues with $\beta_1$- and/or $\beta_2$-ARs, several important metabolic functions are considered to be mediated by the $\beta_3$-AR. As described earlier, $\beta_3$-ARs are known to mediate lipolysis and thermogenesis in adipocytes of various species. More importantly, in non-insulin dependent diabetic models, $\beta_3$-AR mediated activity has demonstrated significant improvement in glucose uptake and insulin sensitivity, generating significant potential for development of drugs to treat obesity as well as diabetes (Smith et al., 1985; Mitchell et al., 1989; Jequier et al., 1992). Several $\beta_3$-AR agonists are undergoing clinical trials for these therapeutic applications (Howe, 1993). Homozygous mutation of Trp64Arg in the $\beta_3$-ARs of obese Pima Indian, Finnish and French subjects has been associated with
obesity and early onset of non-insulin-dependent diabetes in these populations, indicating a role of the β-AR in these disease states (Walston et al., 1995; Widén et al., 1995; Clément et al., 1995). Certain novel β₃-AR agonists have also shown relaxant effects on the gastrointestinal tissues and offer possibility for treatment of diarrhea and other hypermotility disorders (Bianchetti and Manara, 1990; Ford et al., 1992).

The improved β₃-AR affinities and agonist activities of 3'-iodo and 3',5'-diiodo analogs of TMQ have generated the potential for designing TMQ analogs with greater potencies and selectivities for this subtype. Based on differential interactions with the different β-AR subtypes, modifications in the TMQ molecule are directed towards attenuating interactions of the β₁/β₂-subtype such as catechol hydroxyl groups (Konkar, 1996) and towards improving those at the β₃-AR, such as 1-benzyl side chain. Design of potent and β₃-AR selective TMQ analogs would have widespread therapeutic applications in the treatment of obesity, non-insulin-dependent diabetes as well as gastrointestinal hypermotility disorders.
Figure 1. Schematic diagram of β-AR-agonist mediated signal transduction pathway in cells, leading to generation of cAMP and thereby, to biological response. αβγ trimeric protein = G-protein; AC = adenylyl cyclase; GTP, ATP = nucleotide triphosphates; GDP = guanosine diphosphate; R and C units of protein kinase A are regulatory and catalytic subunits of the enzyme, respectively.
Figure 2. Primary structure of the human β3-AR based on the model for rhodopsin. Disulfide bonds are indicated by -S-S-, N-glycosylation sites by Y and the presumably palmitoylated Cys\(^{361}\) residue in the cytoplasmic tail, by a ± (Strosberg and Pietri-Rouxel, 1996).
Figure 3. Chemical structures of some compounds possessing agonist activities on β₁- and/or β₂-ARs. (●) Indicate asymmetric carbon atoms.
Figure 4. Chemical structures of TMQ and some β₂-AR-selective agonists. (•) Indicate asymmetric carbon atoms.
Figure 5. A top view model of agonist binding with the $\beta_3$-AR. (Strosberg and Pietri-Rouzel, 1996).
Figure 6. Chemical structures of selected 1-benzyl substituted derivatives of TMQ including affinity and photoaffinity analogs.
CHAPTER 2

PHARMACOLOGICAL AND AFFINITY STUDIES ON
THE RAT β₃-ADRENOCEPTOR

2.1 Aims

Recombinant expression of receptors offers a convenient and relatively inexpensive system for evaluating basic pharmacological properties of new drug compounds. More importantly, expression of a single receptor subtype in these systems has the added advantage of purity in contrast to native receptors which are generally found to co-exist in tissues with other subtypes. The rat β₃-adrenoceptor (β₃-AR) expressed in Chinese hamster ovary (CHO) cells has been found to exhibit pharmacological properties similar to the atypical β-AR expressed in rat adipocytes (Muzzin et al., 1991). Therefore, this model was used to evaluate the pharmacological properties of a series of TMQ analogs that are being developed as affinity ligands towards characterization and optimization of interactions of this class of compounds with the β₃-AR. Studies were carried out on rat β₃-AR in CHO cells (1) to determine binding affinities, and agonist activities in terms of cAMP
accumulation, of selected 1-benzyl ring and catechol substituted analogs on this receptor subtype, and (2) to determine the affinity characteristics of 4'-α-haloacetamido-3',5'-diodoTMQ analogs. In this latter set of experiments, studies were conducted to determine time and concentration dependence of affinity binding. Protection assays with competing agonists and nucleophiles were conducted to elucidate site-specificity and chemical nature of affinity binding interactions with the receptor subtype.

Functional studies were carried out in isolated rat tissues (1) to quantitate agonist activities of selected analogs on the different β-AR subtypes, as well as the α-ARs, (2) to determine whether the agonist potencies obtained from biochemical studies in the recombinant receptor expression system correlate with those in the functional pharmacological system, and (3) to determine whether the 4'-α-chloroacetamido-3',5'-diodoTMQ analog demonstrates pharmacological response indicative of irreversible binding interactions of the compound.

2.2. Materials and Methods

2.2.1. Chemicals

The chemicals used in studies with CHO cells, and their sources were: Trizma-HCl, (-)-isoproterenol bitartrate, 3-isobutyl-1-methylxanthine, dimethylsulfoxide (DMSO), N-[2-hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid] (HEPES), forskolin, glutathione (reduced form), L-cysteine hydrochloride monohydrate and L-lysine monohydrochloride (Sigma Chemical Co., St. Louis,
MO); L-ascorbic acid (J.T. Baker Chemical Co., Phillipsburg, NJ); (±)-propranolol (Ayerst Laboratories Inc., New York, NY); [\(^{125}\)I]-(-)-3-iodocyanopindolol (2000 Ci/mmol) (Amersham, Arlington Heights, IL); Ham’s F-12 cell culture medium, Hank’s phosphate buffered saline, L-glutamine, penicillin-streptomycin solution, trypsin-EDTA solution, geneticin (G-418) and heat inactivated fetal bovine serum (Gibco, Gaithersburg, MD). BRL 37344 (RR,SS) was a gift from Smith Kline Beecham Pharmaceuticals (Herts, U.K.). TMQ and its analogs were provided by Dr. Duane Miller (Department of Pharmaceutical Sciences, University of Tennessee, Memphis, TN). The chemical structures of the β-AR compounds including TMQ analogs used in these studies are shown in figures 3, 4, 6 and 7, and described in Appendices A and B. All other materials used were of reagent grade. All drugs were dissolved at their highest concentration in double distilled water, except \(10^{-2}\)M forskolin, which was dissolved in dimethylsulfoxide. All subsequent drug dilutions were made in double distilled water for cAMP accumulation studies, and in Tris-EDTA buffer, pH 7.4, for the radioligand binding studies. Tris-EDTA (tris) buffer of following composition was used for the radioligand binding assays: 75 mM Trizma-HCl, 154 mM NaCl and 20 mM EDTA, pH 7.4 in double distilled water.

The chemicals used for functional studies in isolated rat tissues, and their sources were: hydrocortisone sodium succinate (Abbott Laboratories, Chicago, IL), carbamyl choline chloride, i.e. carbachol (Aldrich Chemical Co., Milwaukee, WI), cocaine HCl (Mallinckrodt Chemical Works, St. Louis, MO), (±)-pindolol (Receptor Research Biochemicals Inc., Baltimore, MD), (-)-
isoproterenol-(+)-bitartrate and phentolamine HCl (Sigma Chemical Co., St. Louis, MO). U-0521 (3',4'-dihydroxy-2-methylpropio-phenone) was provided by Dr. Popat N. Patil (College of Pharmacy, The Ohio State University, Columbus OH). The acetamido and chloroacetamido derivatives of 3',5'-diiodoTMQ, (DITMQ, see figure 6 and appendix A) were provided by Dr. Duane D. Miller (Department of Pharmaceutical Sciences, University of Tennessee, Memphis, TN). The drugs were dissolved at their highest concentration (1-10 mM) in double distilled water and subsequent dilutions were also made in double distilled water. All other chemicals used were of reagent grade. Modified Kreb's buffer was used for rinsing and bathing the rat tissues. It was freshly prepared daily and its composition was as follows: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 2H$_2$O, 5.0 mM MgCl$_2$, 6H$_2$O, 1.0 mM NaHPO$_4$, H$_2$O, 25 mM NaHCQ, 11.1 mM dextrose and 26 μM EDTA. Cocaine HCl (3x10$^{-5}$M), hydrocortisone (10$^{-4}$M) and U-0521 (10$^5$M) were added to the Kreb's buffer to inhibit adrenergic neuronal uptake, extraneuronal uptake and to inhibit the enzyme catechol-O-methyl transferase, respectively.

2.2.2. CHO Cells: Source and Culture Conditions.

CHO cells expressing the rat-β$_3$-AR were provided by Dr. Claire Fraser (National Institutes of Health, Bethesda, MD). Culture medium for these cells consisted of Ham's F-12 nutrient medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml-50 μg/ml penicillin-streptomycin, and 500 μg/ml geneticin (used as a selection marker for the cells expressing the rat-β$_3$-AR). The cells were grown and incubated at 37°C in a humidified atmosphere.
of 5% CO₂ : 95% air in a water-jacketed incubator. During each passage, the
cells were rinsed with Hank's phosphate buffered saline followed by trypsin-
EDTA solution and incubated for 5-10 min to allow cells to detach. Cells were
suspended in sufficient volume of culture medium. Portion of the harvested
cell suspension was used to innoculate the next passage (using final dilution
ratio of 1:6). Remaining suspension was centrifuged at approximately 180-
240 x g for 3-4 min, and the pelleted cells were resuspended in tris buffer for
binding studies. Cell number in the suspension was determined using a
hemocytometer, and final concentration was adjusted by addition of more
buffer, where required.

2.2.3. Competitive Radioligand Binding Assays

Competitive radioligand binding assays were performed with whole CHO
cells expressing the rat-β₂-AR by the method of Engel et al. (1981).
[^125]Iodocyanopindolol ([¹²⁵I]ICYP, dissociation constant, K_d = 1.3 nM (Muzzin
et al., 1991), was used at concentrations of 0.8-1.25 nM, as the competing
ligand for the studies. Competitive binding assays involved addition of a fixed
concentration of [¹²⁵I]ICYP and cell suspension (about 500,000 cells) in tris
buffer, to increasing concentrations of competing drug in a total volume of
250 μl. Nonspecific binding was determined in presence of 10⁻⁴ M (±)-
propranolol. The samples were incubated at 37°C for 1 hour for binding
equilibrium. Reactions were terminated by rapid filtration through Whatman
GF/B filters using a Brandel Model 12-R cell harvester, and radioactivity on the
dried filter discs was measured in a Beckman Model 8000 gamma counter.
2.2.4. cAMP Accumulation Studies by Radioimmunoassay

CHO cells expressing the rat-β₂-AR were grown to confluency in 60 x 15 mm sterile dishes. Confluent cultures were washed with 2-3 ml of Hank's phosphate-buffered saline to remove media, secreted metabolites and cell debris. The cells in each dish were then incubated for 30-40 mins at 37°C with 5 ml of modified Hank's buffer containing 1 mM IBMX as the phosphodiesterase inhibitor, 1 mM L-ascorbic acid and 15 mM HEPES, pH 7.4. The buffer-containing cells were then treated with various drugs (each dish treated with a specific concentration of a drug) covering the concentration-response range, and incubated at 37°C for 20 mins. Untreated (basal) and 25 μM forskolin-treated cells were used to determine functional viability (ability to produce an increase in cAMP subsequent to drug treatment), and (-)-isoproterenol at concentrations stimulating maximal accumulation of cAMP was used as the reference standard during each experiment. After the 20 min incubation, the buffer was aspirated, and cells in each dish were treated with 2 ml of 6% trichloroacetic acid (TCA) for 40 min at room temperature, to extract the cAMP. TCA extracts were collected, and the cell protein in each dish was dissolved in 1 ml of 0.1 N NaOH. Protein content of each sample was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Supernatant 0.5 ml fractions of TCA extracts were then extracted three times with 2 ml of water-saturated diethyl ether to remove the TCA from samples. After evaporation of any residual ether phase in a water bath at 50°C for 60-90 min, pH of the samples
was increased to approximately pH 6.2 by adding 0.5 ml of 1M sodium acetate (NaAc) buffer, pH 6.5.

The radioimmunoassay technique of Brooker et al. (1979) was used for cAMP determinations. Samples in 1M NaAc buffer were diluted further with 50 mM NaAc buffer (pH 6.3) to obtain cAMP concentrations (5-500 fmol/100 μl) that would lie within the linear region of a 16 point cAMP standard curve (0.305-10,000 fmol/100 μl in 50 mM NaAc buffer, pH 6.3). Aliquots (100 μl) of samples and standard solutions of cAMP were prepared in duplicate, and acetylated by adding 10 μl of a 2:5 mixture of acetic anhydride: triethylamine, followed by immediate vortexing. The acetylation reaction was allowed to proceed for 20-30 min. Acetylation of cAMP in the samples and standards imparts structural characteristics similar to the competing [125I]-labeled succinyl-cAMP-tyrosine methyl ester used in the assay, resulting in improved affinity of the antibody for the ligand and thereby, increased sensitivity of the assay. The acetylation reaction was quenched by adding 50 μl of 0.25 % bovine serum albumin (BSA) to the samples. 50 μl [125I]-succinyl-cAMP-tyrosine methyl ester (in 0.25 % bovine gamma globulin) and 50 μl cAMP antiserum (in 50 mM NaAc buffer containing 5 % BSA) were then added to each tube, vortexed, and allowed to incubate for at least 16 hrs at 4°C for competitive binding to occur and equilibrate. Finally, the antibody-cAMP complex was precipitated by adding 2 ml of a 60% saturated solution of ammonium sulfate to each tube. The samples were vortexed and kept for 20 min at 4°C to allow complete precipitation and then centrifuged at approximately 2000 x g for 20 min. Supernatant fractions were discarded,
and radioactivity in the precipitates was measured using gamma scintillation counter (Beckman model 8000 gamma counter).

2.2.5. Time and Concentration Dependence of Affinity Binding

Whole CHO cells expressing the rat β3-AR (50-65 x 10⁴ cells/150μl) were suspended in 1.2 ml tris buffer in microfuge tubes and incubated at room temperature in a rotating shaker (Robbins Scientific) with acetamido, chloroacetamido or bromoacetamido analogs of DITMQ at concentrations of 3-, 10-, 30- and 100-K, for time periods ranging from 2 min to 90 min. Incubations were stopped by centrifugation of the cell suspension for 2-3 min at about 1500 x g in a microcentrifuge (Eppendorf Model 5415C). Cell pellets were resuspended in 1.2 ml of fresh buffer, and the samples were placed again on the shaker for about 15 min for bound drug to dissociate, followed by recentrifugation and resuspension. This washing procedure was repeated for a total of three times for each sample. Protein contents of the final reconstituted cell suspensions were determined by the method of Lowry et al. (1951). Triplicate aliquots of normalized suspensions (30,000 to 50,000 cells/aliquot) were incubated with 0.8-1.25 nM of [125I]ICYP in a final volume of 250 μl in buffer for one hr at 37°C. Binding reactions were terminated, and radioactivity was measured as described in an earlier section for competitive radioligand binding. Non-specific binding for each sample aliquot was determined in the presence of 10⁻⁴ M (±) propranolol.
2.2.6. Protection Assays with Competing Agonists and Nucleophiles

Suspensions of CHO cells (1.2 ml) expressing the rat β3-AR in tris buffer were incubated for 20 min at room temperature in a rotating shaker with (-)-isoproterenol, BRL 37344 or acetamidoDITMQ at concentrations 30-fold or 100-fold the respective Kᵢ value. The acetamido or chloroacetamido analog of DITMQ was then added to the suspension and incubated for 2-3 min at molar concentrations, 3-fold or 10-fold of the respective Kᵢ, so that the protecting agent was present at 10-fold higher concentration in terms of Kᵢ compared to the TMQ analog under evaluation. The competitive binding reactions were stopped by centrifugation of the cell suspensions at 1500 x g in a microcentrifuge (Eppendorf Model 5415C), and cells were subjected to 3 washes with buffer as described in the previous section. Normalization of protein content of the reconstituted suspensions and radioligand binding assays were carried out as described previously.

Protection assays were carried out similarly with 10-100 mM glutathione, L-cysteine or L-lysine as the protecting agents. Solutions of the nucleophiles were prepared at pH 7.4 in tris buffer and used on the same day. ChloroacetamidoDITMQ was allowed to interact with the nucleophiles in freshly prepared solution for 20-30 min before addition of cells. After 2-3 min of adding cells, the suspension was centrifuged at 1500 x g for 2 min. Further washing and assay protocol was identical to that described for the competing agonists.

In control studies, washout of the protecting agents (agonists and nucleophiles) was determined by treating cells with these agents in the
absence of affinity ligand and following identical protocol for incubation, washing and radioligand binding.

2.2.7. Preparation of Isolated Tissues.

Male Sprague Dawley rats (Harlan Industries, Cumberland, IN) weighing between 200-430 g were housed under a 12 hr light/dark cycle and fed Purina Rodent Laboratory Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. The animals were killed by cervical dislocation following a blow to the head, and tissues were quickly removed according to standard procedures (Staff of the Department of Pharmacology, University of Edinburgh, 1968). Spontaneously beating right atria, trachea, aorta and esophagus were placed in the modified Kreb's solution warmed to 37°C and gassed with 95% O₂ : 5% CO₂ and were rinsed with the solution every 10-15 mins until measurement of functional response. All drug-mediated tissue responses were measured on a Grass Polygraph Model 7C with a Grass FT-03C isometric force-displacement transducer.

Chronotropic responses of spontaneously beating right atria were used as a model for β₁-AR activity of drugs. The tissue was tied at one end to a glass hook and at other end to the transducer and suspended in 10 ml of gassed buffer in a water-jacketed tissue bath at 37°C under a resting tension of 1 g. The tissue was allowed to equilibrate for 1 hour before measurements.

The relaxation of carbachol-contracted (10⁻⁶M) spirally cut tracheal strips was used for measurement of β₂-AR activity. Generally, two strips were obtained from each animal. Before concentration-response
measurements, the strips were incubated in the water-jacketed bath for 1 hour at a resting tension of 1 g to equilibrate.

The relaxation of carbachol-contracted ($10^{-6}$M) rat esophageal smooth muscle in the presence of 1 $\mu$M pindolol and 10 $\mu$M phentolamine was used as a model for atypical $\beta_3$-AR, and this tissue was prepared by the method of Buckner and Christopherson (1974). Briefly, a 4-5 cm segment of esophagus measured from the diaphragm was removed and cut into longitudinal strips. The smooth muscle layer was carefully separated from the mucosal layer, and the former was tied at one end to the glass hook and at the other end to the transducer, with the tissue immersed in 10 ml buffer in the water-jacketed bath. The tissue was allowed to equilibrate at a resting tension of 200 mg for 1 hour or until stabilization of baseline was achieved.

Contraction of spirally cut strips of rat aorta and inhibition of phenylephrine-induced contraction of the tissue were used to measure $\alpha$-AR mediated agonist or antagonist activity of the compounds, respectively. The aortal strips were equilibrated at a resting tension of 1 g for 2 hours before measuring any functional responses.

### 2.2.8. Measurement of Functional Response

Cumulative concentration-response curves for each drug in the different tissues were constructed as per the method of Van Rossum (1963). Drug concentrations were added every 2-3 min ((-)-isoproterenol or phenylephrine) and every 10-15 min (TMQ analogs) or until no further change in response was observed.
Two concentration-response curves were constructed with each right atrial tissue: first with the reference compound, (-)-isoproterenol, followed by the TMQ analog, after complete washout of the former drug. Drug-mediated chronotropic responses of the right atria were recorded for each incremental addition of drug concentration until a maximal response was achieved. Upon completion of the concentration-response curve with (-)-isoproterenol, the tissue was washed rapidly 7 times with warm buffer (37°C), followed by subsequent washings every 10 min until the response returned to baseline levels. After a further 30 min equilibration of the tissue, a second concentration-response curve with either the acetamido- or chloroacetamido-DITMQ analog was constructed. In some atria, second concentration-response curves were constructed with (-)-isoproterenol in order to determine desensitization effects. Responses to the TMQ analogs were calculated based on the maximal response to (-)-isoproterenol.

The tracheal strips were contracted with $3 \times 10^{-7}$ M carbachol to induce a response 60-70% of the maximum, and the response was allowed to stabilize (10-15 min). Cumulative concentration-relaxation response curves were constructed for the drug on this precontracted tissue. Finally, a $10^{-5}$M dose of (-)-isoproterenol was added to determine the maximal relaxation induced in the tissue. Each tracheal strip was used for one dose-response curve. Strips precontracted with carbachol were included as time controls for drug-induced relaxation response, through the duration of study.

The esophageal smooth muscle strip was first equilibrated with 10 $\mu$M phentolamine and 1 $\mu$M pindolol for 30-45 min to block the $\alpha$- and $\beta_1/\beta_2$-ARs,
respectively, followed by treatment with 1 μM carbachol to induce 70-80% maximal contraction. The contraction peaked within 10-15 min, followed by some fade that converted into a sustained plateau within 30 min. Cumulative concentration-relaxation responses were then constructed on this tissue. Maximal relaxation response was measured finally by addition of a single concentration of 10^-9 M (-)-isoproterenol.

In all the above three tissues, drug-induced effects were expressed as a percentage of the maximal response to (-)-isoproterenol.

Studies with the rat aorta strip were carried out following incubation for 30 min with 1 μM pindolol to block β-AR mediated effects. The tissue was then contracted with increasing concentrations of phenylephrine (10^-9-10^-5 M), and a cumulative concentration-response curve was generated. Upon reaching a maximal response, the tissue was washed rapidly several times with Kreb's buffer (7 immediate washes followed by one every 10 min) until basal tension levels were re-established, which took generally 30 min. 1 μM Pindolol was added again to the bath, and the aorta was allowed to incubate for 30 min. In order to determine any α-AR mediated effect (agonist/antagonist), acetamido- or chloroacetamido-DITMQ was added at a concentration of 10^-5 M to the tissue bath. An agonist effect would be indicated by a contractile response of the tissue. To check for an antagonist effect, the aorta was allowed to incubate in the drug-containing bath for 30 min, and a phenylephrine concentration-response curve was again constructed. A rightward shift in the curve or suppression of the maximal response to phenylephrine subsequent to drug treatment would be indicative of α-AR
antagonist effect of the drug. Phenylephrine concentration-response curves were constructed in the absence of any drug treatment, as controls for these studies.

2.2.9. Duration of Response following Washout of Compounds.

Following construction of each concentration-response curve in the right atria, the tissue was washed immediately 7 times with the buffer, followed by subsequent washings every 10 min until the heart rate returned to baseline levels. Identical washing procedure was followed subsequent to treatment with (-)-isoproterenol as well as the TMQ analogs. Responses were measured periodically to determine the average washout time required for the heart-rate to decrease from maximal levels to the basal levels.

2.2.10. Data Analysis

Radioligand binding data were analyzed using GraphPAD Inplot version 3.14 (GraphPAD Software Inc., San Diego, CA). Single-site non-linear regression analysis of specific binding yielded 50% inhibitory concentration (IC₅₀) values, from which dissociation constants of competing ligands (Kᵢ) were determined using the equation of Cheng and Prusoff (1973) as follows:

\[ Kᵢ = \frac{IC₅₀}{IC₅₀ + [L]/K_l} \]

where, \( IC₅₀ \) = molar concentration of competing drug displacing 50% of specific receptor-bound radioligand,

\( [L] \) = molar concentration of the radioligand,

\( K_l \) = dissociation constant of radioligand for the receptor.
Percentage receptor occupancy of the various ligands was calculated using the equation of mass law of action:

\[
\% \frac{[R_A]}{[R_T]} = \frac{[D] \times 100}{K_i + [D]}
\]

where, \([R_A]\) = number of receptors occupied by ligand;
\([R_T]\) = total number of receptors;
\([D]\) = molar concentration of the drug "D" around receptor site; and
\(K_i\) = dissociation constant of the drug for the receptor (as determined from competitive radioligand binding assays).

For cAMP radioimmunoassay, the percent bound radiolabeled cAMP was determined for the standards and samples from the following equation:

\[
\% B = \frac{cpm \times 100}{(Bo - Bk)}
\]

where cpm = counts per minute of radiolabeled cAMP bound to antibody;
\(Bo = cpm\) of total antibody-bound radiolabeled cAMP in absence of any competing cold cAMP; and
\(Bk = cpm\) of background radioactivity associated with the test-tube in the absence of cAMP antibody.

cAMP concentration in samples was determined by interpolating values from a 16-point standard curve generated using the GraphPAD Inplot version 3.4. Nonlinear regression analysis of the individual concentration-response curves yielded agonist concentrations producing a half-maximal response in terms of cAMP accumulation \((EC_{50})\), which were also expressed as \(pK_{ec}\) or negative log of \(EC_{50}\). Maximal cAMP accumulation for each agonist was also
obtained from the concentration-response curves and was expressed relative to maximal cAMP accumulation produced by (-)-isoproterenol.

Relative efficacies \( (e_i) \) values were obtained from plots of cAMP accumulation versus percent receptor occupancy as described by Furchgott and Bursztyn (1967). Relative efficacy was calculated as the ratio of percentage receptors occupied by the reference and test agonists in producing half-maximal cAMP accumulation levels. (-)-Isoproterenol was used as the reference agonist for these studies.

The unpaired Student's t-test was used to determine statistical difference at a significance level of 5%.

2.3. Results

2.3.1 Binding Affinities of TMQ Analogs

Concentration-dependent competition of specific binding of \( ^{125}\text{I} \)ICYP from the rat-\( \beta_3 \)-AR was demonstrated by the various \( \beta \)-AR ligands including (-)-isoproterenol, BRL 37344 and the TMQ analogs (Figure 8). The binding affinities of these ligands for the receptor, denoted as the negative log values of the inhibitory constants (pK\(_I\)), were determined from the competition curves and are shown in Table I. The non-specific binding to these receptors varied between 10-25% in these experiments. The values of K\(_I\) obtained with the GraphPAD analysis coincided with those obtained from the PC-version of LIGAND, the radioligand binding program that had been used previously in our laboratory and which indicated single-site ligand binding i.e. to a homogenous
receptor population. The rank order of potencies for inhibiting ICYP binding were as follows (pKᵢ values in parentheses): acetamidoDITMQ (7.28) > BRL37344 (6.96) > bromoacetamidoDITMQ (6.70) > chloroacetamidoDITMQ (6.49) > DITMQ (6.34) > aminoDITMQ (6.14) ≥ isothiocyanatolTMQ (5.83) ≥ demethoxyDITMQ (5.67) > S(-)TMQ (5.67) > 2-(naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (5.39) ≥ (±)TMQ (5.11) ≥ 1-(naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (5.07) > 6,7-dimethoxyacetamidoDITMQ (4.84) > (-)-isoproterenol (4.45) ≥ 6,7-methylenedioxyTMQ (4.29) > 6,7-dimethoxyTMQ (3.88). With the exception of the 6,7-dimethyl or methylenedioxy substituted analogs of TMQ, all the compounds, including the parent compound TMQ and its analogs with substituents on the 1-benzyl ring, displayed higher affinities for the rat β₃-AR as compared to (-)-isoproterenol. In general, the affinity increased with iodo substitution at the 3'- and 5'-positions of the benzyl ring of TMQ, as well as with increasing lipophilicity and bulk at the 4'-position of the ring. Binding affinities of acetamido and haloacetamido derivatives were over 100-fold higher as compared to (-)-isoproterenol, and were comparable to that of the rat β₃-AR selective ligand, BRL 37344. Among the TMQ analogs studied, the acetamidoDITMQ displayed highest affinity for the receptor, which was 2-fold higher than that of BRL 37344. Aryl substitutions at the 3',4'- or 4',5'-positions of the benzyl side-chain did not improve receptor affinity. Replacing the catechol groups on the parent compound with methylenedioxy groups decreased the binding affinities for the receptor by 24-fold while replacements with dimethoxy groups decreased the affinities by 61- to 275-fold.
2.3.2 Biochemical Potencies of TMQ Analogs

The functional ability of selected TMQ analogs and standard compounds to generate cAMP in the CHO cells through activation of the rat β3-AR was concentration-dependent (figure 9). The biochemical potencies of these compounds, denoted as the negative log of concentrations eliciting half-maximal cAMP accumulation (-log EC$_{50}$ or pK$_{act}$), as well as the maximal cAMP accumulations relative to that of (-)-isoproterenol (E$_{\text{max}}$) are summarized in Table I. The basal levels of cAMP accumulated in these cells were 103 ± 22 pmoles/mg protein, while 25 μM forskolin-stimulated levels were 5460 ± 1050 pmoles/mg protein (mean ± SEM). The maximal cAMP accumulation induced by (-)-isoproterenol treatment (E$_{\text{max}}$ = 100%) was 2260 ± 390 pmoles/mg protein. The rank order of potencies of the compounds in decreasing order were as follows (pK$_{act}$ in parentheses): DITMQ (9.40) ≥ bromoacetamidoDITMQ (9.36) ≥ acetamidoDITMQ (9.34) ≥ chloroacetamidoDITMQ (9.05) ≥ BRL 37344 (8.90) ≥ demethoxyDITMQ (8.74) ≥ (±)TMQ (8.69) ≥ isothiocyanatolTMQ (8.61) ≥ S(-)TMQ (8.19) ≥ (-)-isoproterenol (7.90). BRL 37344, TMQ and the selected analogs, with the exception of 6,7-methylenedioxyTMQ (a partial agonist), exhibited full agonist activity. In addition, 6,7-dimethoxyTMQ exhibited no activity at a concentration of 3x10$^{-5}$M. Thus, replacing the catechol moiety of TMQ with methylenedioxy or dimethoxy moieties significantly attenuated or abolished the agonist activity of the compound. On the other hand, 3',5'-diiodo substitution on the benzyl side-chain increased the functional potency of the compound, and 4'-acetamido- or 4'-α-haloacetamido-substituted analogs more
or less retained these potencies. These diiodo derivatives of TMQ were more potent than the reference rat-β3-AR-selective agonist, BRL 37344. IsothiocyanatoTMQ was also a full agonist, and although its potency was lower compared to those of the diiodo derivatives, it was comparable to the biochemical potency of the parent compound, TMQ. The potencies of these benzyl substituted TMQ analogs were 5-30 fold higher than that of the standard agonist, (-)-isoproterenol.

Plots of receptor occupancy versus cAMP accumulation of selected compounds are shown in figure 10. (-)-Isoproterenol occupied the least number of receptors to elicit 50% biochemical response (0.0283%). The other compounds including BRL 37344 and the benzyl substituted TMQ analogs occupied larger fractions of the receptors to produce an equivalent response. The efficacies (e,) of these compounds relative to (-)-isoproterenol (e, = 1.00) in decreasing order were: isothiocyanatoTMQ, 0.20 (0.028/0.145) > bromoacetamidoDITMQ, 0.118 (0.028/0.26) > (-)TMQ, 0.059 (0.028/0.48) > chloroacetamidoDITMQ, 0.047 (0.028/0.60) > acetamidoDITMQ, 0.030 (0.028/0.94) > BRL 37344, 0.022 (0.028/1.27). Thus, the TMQ analogs occupied significantly higher fractions of receptors for equivalent activity as compared to (-)-isoproterenol, but lower as compared to BRL 37344.

2.3.3. Effect of Incubation Time and Ligand Concentration on Affinity Binding

When CHO cells expressing the rat-β3-AR were incubated for 40-45 min with the acetamido, chloroacetamido or bromoacetamido derivatives of
DITMO at concentrations ranging from 3- to 100-$K_i$, inhibition of specific binding of [$^{125}I$]ICYP to the receptors was observed after washout of the compounds (see figure 11). This inhibition of [$^{125}I$]ICYP binding, indicating "irreversible" binding of the compounds to the rat-$\beta_3$-AR, was dependent upon the concentrations of the haloacetamido derivatives used. The chloroacetamido derivative was the most effective affinity ligand, inhibiting the radioligand binding to the receptors in a concentration-dependent manner by 50-90%. By contrast, interestingly, the bromoacetamido derivative inhibited specific ICYP binding to the receptors by only 15-50% with increasing concentrations. The acetamido analog did not influence radioligand binding to the receptors significantly (except at very high concentrations of 100$K_i$ when about 20-25% inhibition of ICYP binding was observed), indicating that the nature of binding for this compound was reversible in nature. Only at 100$K_i$ molar concentration of the acetamido derivative, a slight decrease in inhibition of ICYP binding was observed, suggesting the possibility of incomplete washout of the lipophilic compound at high concentrations.

The effect of incubation time on the irreversible binding of these compounds was studied at concentrations of 3- and 10-$K_i$ (see figure 12). While a very slight decrease in ICYP binding to the rat-$\beta_3$-AR was observed with increasing periods of incubation with the bromoacetamido derivative, no significant change in binding with time was observed with the chloroacetamido compound.
2.3.4. Protection Assays with Competing Agonists and Nucleophiles.

In order to investigate the site of irreversible binding of chloroacetamidoDITMQ to the rat $\beta_3$-AR, protection assays were carried out with the non-specific $\beta$-AR agonist, (-)-isoproterenol; the $\beta_3$-AR specific agonist, BRL 37344; and acetamidoDITMQ, a close structural agonist analog of the affinity ligand. As controls for these studies, similar treatments with the protection agents were carried out on the reversible binding of acetamidoDITMQ analog. The inhibition of ICYP binding to the receptors with these ligands in the absence and presence of protecting agents is summarized in figure 13. While the chloroacetamido derivative inhibited the ICYP binding in a concentration dependent manner, this binding was not affected by any of the competing agonists. This indicated failure of these $\beta$-AR agonists to protect against the irreversible binding of the affinity ligand to the receptors. When cells were treated similarly with the acetamido derivative in the presence or absence of protecting agents, ICYP binding to the receptors was not significantly reduced, indicating complete washout of the reversibly binding agonists from the cells. This was further confirmed by separate washout studies with the protecting agents, as shown by the results in figure 14. There was no significant difference in ICYP binding to the cells subjected to 3 and 5 washes after treatment with the agonists, indicating that three washes in the experimental protocol were sufficient to remove reversibly-bound ligands from the receptors. In these experiments, washout of the reversibly bound agonists was indicated by over 80% specific binding of ICYP.
to the receptors, whereas the chloroacetamido analog of TMQ, used as a positive control, inhibited the ICYP binding to a much greater extent.

Similar protection experiments with 10 and 100 mM concentrations of glutathione, L-cysteine and L-lysine were carried out to determine the nature of chemical interactions of the affinity ligand, chloroacetamidoDITMQ with the rat β3-AR. As shown in figure 15, the affinity compound inhibited ICYP binding to the receptors as usual in a concentration-dependent manner. This inhibition was not attenuated in the presence of these three nucleophiles, indicating that none of these agents were able to quench the reactivity of the 4'-α-chloroacetamido group of the affinity analog of TMQ with the rat β3-AR.

Figure 16 summarizes the effects of preincubation with the various competing agonists and nucleophiles on the binding of chloroacetamidoDITMQ to rat β3-ARs. Extent of protection offered was determined by the net increase in specific-[125I]ICYP binding to the receptors upon treatment with the affinity agent in presence of the protecting agent. Of all the compounds studied, only L-lysine demonstrated a small, but insignificant protective effect.

### 2.3.5. β-AR Mediated Functional Activities in Rat Tissues

Concentration-dependent agonist activities of (-)-isoproterenol, acetamidoDITMQ and chloroacetamidoDITMQ in right atria, trachea and esophageal smooth muscle of rat are shown in figure 17, and the agonist potencies as well as the intrinsic activities of the compounds are summarized in Table II. All three compounds exhibited positive chronotropic effects on right atria with nearly equipotent activity (EC50 of 1.09-1.15 nM). However,
the TMQ analogs were partial agonists on this tissue (intrinsic activity, I.A. of 0.93 and 0.81 with the acetamido and chloroacetamido derivatives, respectively), relative to (-)-isoproterenol (I.A. of 1.00). An average of a 2-fold shift to the right was observed in the potency of (-)-isoproterenol between the first and second concentration-response curves on each tissue. These tissue-desensitization effects were corrected for determination of the potencies of the TMQ compounds.

In contrast to the equipotent effects in right atria, the TMQ analogs produced significantly more potent relaxation compared to (-)-isoproterenol, in partially contracted trachea and esophageal smooth muscle preparations. The potencies (EC_{50}) of the compounds in decreasing rank order were 0.6 and 2.1 nM for acetamidoDITMQ > 1.3 and 8.3 nM for chloroacetamidoDITMQ > 10 and 46 nM for (-)-isoproterenol, in the trachea and esophageal smooth muscle, respectively. Thus, the acetamido derivative was the most potent of the compounds studied, and both TMQ derivatives were 8-16 fold more potent in the trachea, and 6-22 fold more potent in the esophageal smooth muscle in comparison to (-)-isoproterenol. However, while the TMQ derivatives were partial agonists in the tracheal tissue (I.A. around 0.83-0.84 ± 0.02), they were full agonists in the esophageal smooth muscle (I.A. of 0.99 ± 0.03).

An interesting observation in these studies, was the response time of the tissues following addition of each drug concentration to the tissue bath. While the tissue responses were observed within 2-3 min following addition of (-)-isoproterenol, it took much longer (10-20 min) to observe a response.
following treatment of tissues with varying concentrations of the TMQ analogs.

2.3.6. Duration of Chronotropic Response in Rat Right Atria Following Washout.

The washout time of the agonists from the right atrial tissue was indicated by the time required for the maximal heart-rate to return to basal levels during a series of washouts in the tissues. Figure 18 illustrates the duration of chronotropic response in the atria using identical washout protocols following treatment with the different agonists. The responses mediated by (-)-isoproterenol and acetamidoDITMQ subsided completely after an average of 70-75 min following repeated washings at regular intervals (10-15 min), whereas chloroacetamidoDITMQ-mediated responses persisted for a significantly greater period of time. Greater than 10% of the initial agonist activity was retained at the end of 3 hours and 20 min. The average times for half recovery to 50% of the drug's maximal chronotropic effect were 14 and 17 mins for (-)-isoproterenol and acetamidoDITMQ, respectively, whereas in the case of chloroacetamidoDITMQ the average half-time was 95 min.

2.3.7 Functional Activity in Rat Aorta.

Concentration-response curves generated in the rat aorta with the α-AR agonist, phenylephrine, before and after incubation of the tissues with the TMQ analogs, are shown in figure 19. Phenylephrine produced a concentration-dependent contraction of the aorta which was not modified by
preincubation of the tissue with $10^{-6}$M concentration of either the acetamido or chloroacetamido analog of DITMQ. A small rightward shift in the concentration-response curves of phenylephrine was observed following incubation of the tissue with the TMQ compounds. However, this small shift in the concentration response curve of phenylephrine was not significantly different from the controls when tissue desensitization effects were considered. The TMQ analogs by themselves, did not produce any contraction of the aorta tissue at the highest concentrations used. These studies indicate that the TMQ compounds lack $\alpha$-AR mediated agonist and antagonist activity in the rat.

2.4 Discussion

The binding affinities and biochemical potencies of the 1-benzyl substituted TMQ analogs on rat $\beta_3$-ARs in CHO cells were higher than those of the standard $\beta$-AR agonist, (-)-isoproterenol, and were comparable to those of the highly potent $\beta_3$-AR-selective agonist, BRL 37344. In comparison to the parent compound, TMQ, the 1-benzyl substituted analogs of TMQ possessed higher binding affinities and biochemical potencies on the rat $\beta$-AR, and these properties improved significantly with the addition of lipophilic and bulky substituents on the 3',4',5'-positions of the 1-benzyl side chain. These results indicate the importance of the substituents on the benzyl group, and are in agreement with Blin's hypothesis (Blin et al., 1993) that the bulky, lipophilic side chain of $\beta_3$-AR agonists may be contributing significantly to their agonist activities on the receptor by interacting at a site different from that of
the catechol site of interaction. Diiodo substitution at the 3'- and 5'-positions of the 1-benzyl ring of TMQ adds considerable bulk and increases lipophilicity of the compound. Acetamido substitutions at the 4'-position further contribute to increase these properties, which translate into improved binding affinities.

Replacement of catechol hydroxy groups with dimethoxy or methylenedioxy groups abrogated the affinities and agonist properties of the compound, indicating that these substitutions may be perturbing the spacial and/or electronic aspects of the ligand-receptor interactions. Interestingly, weak agonist activity was retained in those compounds in which the catechol group was masked. In this regard, it is known that atypical β/β3-ARs agonists lack the presence of an intact catechol moiety (Strosberg and Pietri-Rouzel, 1996). In the atypical β/β3-AR agonists, the catechol is generally replaced with bioisosteric groups containing chloro or an equivalent functional group on a phenyl or larger aryloxy moiety.

The biochemical potencies of (-)-isoproterenol, acetamido- and chloroacetamido-DITMQ correlated fairly well with their functional potencies for relaxing the esophageal smooth muscle (pK_{act}s of 7.90, 9.34, 9.05, and EC_{50}s of 7.34, 8.68, 8.08, for the three compounds, respectively) indicating that the recombinantly expressed receptors provided a convenient and fairly good substitute for the native receptors under physiological conditions. Although the acetamido- and chloroacetamido-DITMQ had lower relative efficacies as compared to (-)-isoproterenol in the recombinant system (as seen from the receptor occupancy plots) these were higher compared to BRL
37344, and the TMQ compounds exhibited full agonist activities in functional studies. The differences in biochemical and functional potencies of the compounds, as well as differences in maximal activities, may be explained by differences in amounts of spare receptors in the two systems as well as by different end-points of response measurement in the signal transduction pathway. In summary, the acetamido- and haloacetamido-DITMQ analogs were more potent agonists on the rat $\beta_3$-AR than (-)-isoproterenol and were comparable to BRL 37344.

An objective of this research was to examine the irreversible binding of the haloacetamido TMQ analogs to rat $\beta_3$-AR in CHO cells. In reversible competition experiments, the chloroacetamido and bromoacetamido derivatives of DITMQ inhibited the binding of $[^{125}I]$ICYP to rat $\beta_3$-AR with affinities of 323 and 200 nM, respectively, which are comparable to that of BRL 37344 (110 nM). In washout experiments, indirect evidence for covalent interaction of these compounds was presented by the fact that $[^{125}I]$ICYP binding activity was not restored to control levels despite extensive washing of the cells. More importantly, the degree of receptor blockade by these compounds was concentration-dependent. In these studies, the chloroacetamido derivative was a more effective affinity agent than the bromoacetamido analog. Since stability studies (see Appendix C) indicated that both of these compounds were stable in aqueous or methanolic solutions, it is possible that the bromoacetamido derivative could be reacting additionally to a greater extent than the chloroacetamido TMQ analog with non-specific sites on the receptor protein. Moreover, time dependence studies suggested
that the kinetics for affinity binding of these analogs to the receptor were very rapid (<2 min). This may not be surprising, considering that the nucleophilic attack by the haloacetamido moiety is a one-step reaction versus covalent binding of alkylamines which involves generation of an intermediate reactive species (Sweet and Murdock, 1987; Triggle, 1965). In the latter case, incubations of phenoxybenzamine for longer than 10 min are required to achieve maximal inactivation of α-ARs.

The inability of exogenous nucleophilic reagents such as glutathione, L-cysteine and L-lysine to quench the reactivity of the TMQ affinity analog in protection assays failed to offer convincing evidence for covalent binding of the compound to the receptors via nucleophilic substitution. However, in several similar studies involving iodonaphthylazide labeling of intrinsic membrane proteins like cytochrome oxidase, Ca-ATPase, (Na,K)-ATPase and glycophorin, the presence of glutathione in the aqueous phase failed to offer any protection. This is in contrast to the findings with the much less hydrophobic phenylazide label (Jørgensen et al., 1982). By analogy, it is possible that the nucleophiles, being relatively polar, were poor competitors for the highly reactive lipophilic affinity compound that is postulated to be occupying the "hydrophobic" binding site in the transmembrane domain of the β3-AR.

However, the lack of protection by competing agonists also strongly suggests that the affinity interaction of our compound may be located at a site outside of the agonist binding pocket, but at a site that may be more in common with the occupation site of ICYP. In this regard, the β2/β3-AR
agonist, salmeterol, is proposed to bind to an exo-receptor site in a non-competitive manner (Johnson et al., 1993). Salmeterol possesses an extended N-alkyl substituent which is predicted to be located deep into a hydrophobic core domain of the receptor which represents the specific exosite, while the saligenin head interacts with the receptor in a manner analogous to salbutamol and other conventional β-AR agonists. The high-affinity binding of the side chain to the exo-site allows the saligenin head to activate the receptor in a continuous manner, enabling salmeterol to exhibit long-acting pharmacological effect. TMQ, like salmeterol, possesses an extended N-alkyl side-chain which may be binding to a hydrophobic exo-site on the β_3-AR in a non-competitive manner. The persistent chronotropic response in the washout phase of atria treated with chloroacetamidoDITMQ provides strong support for the irreversible interactions of the compound with the β-AR. Besides, functional evidence has been utilized in the past as an indicator of affinity binding (Cooperman, 1988).

Recently, by photoaffinity labeling of the β_2-AR with antagonists possessing the photoactivable group either on the aryloxy end or the amino end of the molecule, it was shown that the aryloxy portion of β-AR antagonists is highly constrained within tms 6 and 7 of the receptor, whereas the amino terminus is much less constrained and able to assume multiple conformations (Hockerman et al., 1996). The antagonist derivatives containing a photoactivable group on the amino end derivatized amino acids to a greater extent on tm1, 2, 6 and 7, indicating that a folded conformation of the molecule is favored. Several β_1-β_2-AR antagonists including ICYP are
known behave as partial agonists on the $\beta_3$-AR (Strosberg, 1995). The proposed model of antagonist conformation and interaction with tms6 and 7 of the $\beta$-AR may provide an explanation for the partial agonist behavior of several $\beta_1$-$\beta_2$-AR agonists on the $\beta_3$-AR, as well as for the non-conventional interactions of the ether-containing alkylamine side chain of salmeterol. It also provides added credibility to Blin’s hypothesis of a hydrophobic binding pocket on tms1, 2 and 7 of the $\beta_3$-AR. Likewise, the affinity binding profile of chloroacetamidoDITMQ seems to favor the explanation that the compound interacts with amino acids on tms1, 2, 6 and 7 of the $\beta$-AR and thereby interferes with the binding of ICYP, but this site is not competed by classical agonists. The rapid kinetics of affinity binding of the compound may provide additional explanation for the lack of protection observed with the $\beta_3$-AR agonist, BRL 37344, as well as by the close structural analog, acetamidoDITMQ. Notably, the chloroacetamidoTMQ analog did not interact functionally with the tm7 domain of $\alpha$-AR in rat aorta, suggesting that the interactions are specific for the rat $\beta_3$-AR. Further studies with subtypes of $\beta$-AR will be required to establish the degree of receptor selectivity for potent agonist affinity TMQ analog, and the use of a radiolabel to study the covalent binding followed by proteolysis of the receptor protein will be required to identify the site-specific interactions of this class of novel agonist molecules.
Figure 7. Chemical structures of selected TMQ analogs evaluated for pharmacological activity on the rat β3-AR expressed in CHO cells.
Figure 8. Concentration-dependent inhibition of specific \[^{[125]}I\]ICYP binding to rat \(\beta_3\)-AR in CHO cells by \(\beta\)-AR ligands and TMQ analogs. Values are expressed as the mean ± SEM of \(n = 5-14\) experiments. Key. Panel A: (−)-isoproterenol (□); (±) TMQ* (■); BRL 37344 (∗); DITMQ* (∗); acetamidoDITMQ (●). Panel B: (±) TMQ* (■); demethoxyDITMQ (∆); aminoDITMQ (∗); chloroacetamido-DITMQ (∗); bromoacetamidoDITMQ (∗). * Unpublished data from Paul Fraundorfer.
Figure 9. Concentration-dependent increases in cAMP accumulation in CHO cells upon agonist-mediated stimulation of the rat β3-AR. The data are expressed as the mean ± SEM of 4-14 experiments. Key. Panel A: (-)-isoproterenol (□); (-) TMQ (■); BRL 37344 (○); DITMQ* (△); 6,7-methylenedioxyTMQ (●). Panel B: (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamidoDITMQ (○); bromoacetamidoDITMQ (♦); isothiocyanatoTMQ (■). * Unpublished data from Paul Fraundorfer.
Figure 10. Agonist-mediated cAMP accumulation in CHO cells expressing the rat β<sub>3</sub>-AR as a function of receptor occupancy by selected compounds. Key: (-)-isoproterenol (○); isothiocyanatolTMQ (■); BRL 37344 (○); acetamidoDITMQ (●); chloroacetamido-DITMQ (◇).
<table>
<thead>
<tr>
<th>Compound</th>
<th>pK&lt;sub&gt;i&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;act&lt;/sub&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)-Isoproterenol</td>
<td>4.45 ± 0.06 (14)</td>
<td>7.90 ± 0.12 (16)</td>
<td>100</td>
</tr>
<tr>
<td>BRL 37344</td>
<td>6.96 ± 0.08 (5)</td>
<td>8.90 ± 0.12 (9)</td>
<td>103.3 ± 6.80</td>
</tr>
<tr>
<td>S(−)TMQ</td>
<td>5.67 ± 0.03 (5)</td>
<td>8.19 ± 0.19 (7)</td>
<td>87.65 ± 6.90</td>
</tr>
<tr>
<td>(±)TMQ</td>
<td>5.11 ± 0.12 (8)*</td>
<td>8.69 ± 0.14 (10)*</td>
<td>125.3 ± 9.20*</td>
</tr>
<tr>
<td>DITMQ</td>
<td>6.34 ± 0.03 (5)</td>
<td>9.40 ± 0.08 (9)*</td>
<td>96.90 ± 10.10*</td>
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<td>AminoDITMQ</td>
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<td>nd</td>
</tr>
<tr>
<td>AcetamidoDITMQ</td>
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<td>9.34 ± 0.12 (6)</td>
<td>89.57 ± 6.93</td>
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<tr>
<td>ChloroacetamidoDITMQ</td>
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<tr>
<td>BromoacetamidoDITMQ</td>
<td>6.70 ± 0.05 (6)</td>
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<td>87.27 ± 12.21</td>
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<tr>
<td>6,7-Dimethoxy-acetamidoDITMQ</td>
<td>4.84 ± 0.03 (5)</td>
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<td>nd</td>
</tr>
<tr>
<td>6,7-DimethoxyTMQ</td>
<td>3.88 ± 0.07 (4)</td>
<td>no activity upto 3 x 10&lt;sup&gt;−5&lt;/sup&gt; M</td>
<td></td>
</tr>
<tr>
<td>6,7-MethylenedioxyTMQ</td>
<td>4.29 ± 0.05 (5)</td>
<td>5.92 ± 0.12 (8)</td>
<td>70.90 ± 4.41**</td>
</tr>
<tr>
<td>DemethoxyDITMQ</td>
<td>5.80 ± 0.03 (5)</td>
<td>8.74 ± 0.10 (4)</td>
<td>97.69 ± 5.85</td>
</tr>
<tr>
<td>IsothiocyanatoDITMQ</td>
<td>5.83 ± 0.12 (4)</td>
<td>8.61 ± 0.15 (4)</td>
<td>104.1 ± 7.10</td>
</tr>
<tr>
<td>1-(Naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>5.07 ± 0.05 (5)</td>
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<td>nd</td>
</tr>
<tr>
<td>2-(Naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>5.39 ± 0.05 (4)</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Paul F. Fraundorfer, unpublished data  ** at highest concentration used (3 x 10<sup>−5</sup> M)

Table 1. Inhibition constants (-log K<sub>i</sub> or pK<sub>i</sub>) for binding and functional activity constants (-log EC<sub>50</sub> or pK<sub>act</sub>) for cAMP accumulation in CHO cells expressing rat β<sub>3</sub>-AR. E<sub>max</sub> is the maximal cAMP accumulation stimulated by the compounds relative to that of (−)-isoproterenol. Values are mean ± SEM of the number of experiments indicated in parentheses. nd = value not determined.
Figure 11. Concentration-dependent inhibition of $[^{125}\text{I}]$ICYP binding to rat $\beta_3$-AR in CHO cells in washout experiments after incubation for 40-45 min with acetamidoDITMQ (●), chloroacetamidoDITMQ (○) or bromoacetamidoDITMQ (♦). Values are expressed as the mean ± SEM of 4 experiments, each in triplicate.
Figure 12. Effect of time of incubation with ligands at molar concentrations of 3$K_i$ (open bars) and 10$K_i$ (diagonal bars) in washout experiments, upon the inhibition of ($^{125}$I)ICYP binding to rat $\beta_3$-AR in CHO cells. The data are expressed as the mean ± SEM of 2-4 experiments, each in triplicate. Panel A: ChloroacetamidoDITMQ Panel B: AcetamidoDITMQ Panel C: BromoacetamidoDITMQ.
Figure 13. Effect of (-)-isoproterenol, BRL 37344 and acetamidoDITMQ as competing agents on the concentration-dependent inhibition of \[^{125}\text{I} \]ICYP binding by chloroacetamidoDITMQ (and in control experiments, by acetamidoDITMQ) on rat \( \beta_3 \)-AR in CHO cells. The data are expressed as the mean ± SEM of 3 experiments, each in triplicate. Key. ChloroacetamidoDITMQ (open bars), chloroacetamidoDITMQ + 10-fold \( K_i \) molar excess of protecting agent (forward diagonal bars), acetamidoDITMQ (reverse diagonal bars) and acetamidoDITMQ + 10-fold \( K_i \) molar excess of protecting agent (checkered bars). The protecting agents were: Panel A: (-)-Isoproterenol, Panel B: BRL 37344, and panel C: AcetamidoDITMQ.
Figure 13.
Figure 14. Binding of $[^{125}I]$ICYP to rat $\beta_3$-AR in CHO cells after 3 and 5 washes of cells incubated with selected agonists. Key. 10K$_i$ molar concentration of chloroacetamido-DITMQ for 2-3 min (open bars); 100 K$_i$ molar concentration of (-)-isoproterenol for 20-30 min (forward diagonal bars); or with BRL 37344 for 20-30 min at molar concentrations of 30 K$_i$ (reverse diagonal bars) or 100K$_i$ (checkered bars). The data are expressed as the mean ± SEM of 2-3 experiments, each in triplicate.
Figure 15. Effect of nucleophiles on the concentration-dependent inhibition of $^{[125]}$ICYP binding by chloroacetamidoTMQ to rat $\beta_3$-AR in CHO cells. The data are expressed as the mean ± SEM of 3 experiments, each in triplicate. Key. ChloroacetamidoDITMQ (open bars); chloroacetamidoDITMQ + 0.01 M nucleophile (forward diagonal bars); chloroacetamidoDITMQ + 0.1 M nucleophile (reverse diagonal bars); 0.01 M nucleophile only (horizontal bars); 0.1 M nucleophile only (checkered bars). Panel A: Glutathione, Panel B: L-Cysteine, Panel C: L-Lysine.
Figure 16. Summary of protecting effect of competing agonists and nucleophiles against affinity binding of chloroacetamidoDITMQ (10K) to rat $\beta_3$ AR in CHO cells. Protection is indicated by a positive value of % change in total specific-[125I]ICYP-bound receptors, whereas negative values indicate lack of protection. The values are expressed as the mean ± SEM of n = 3, each in triplicate.
Figure 17. Concentration-dependent functional responses of (-)-isoproterenol (□), acetamidoDITMQ (●) and chloroacetamidoDITMQ (◇) mediated by the different β-AR subtypes in isolated rat tissues. The data are expressed as the mean ± SEM of 4-12 experiments. Panel A: Chronotropic response in right atria (β₁-AR); Panel B: Relaxation of partially contracted tracheal strips (β₂ - AR); and Panel C: Relaxation of partially contracted esophageal smooth muscle (atypical-β/β₃-AR).
Figure 17.
<table>
<thead>
<tr>
<th></th>
<th>Right Atria (β₁-AR)</th>
<th>Trachea (β₂-AR)</th>
<th>Esophageal Smooth Muscle (Atypical-β₁/β₂-AR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Isoproterenol</td>
<td>8.95 ± 0.06</td>
<td>8.00 ± 0.05</td>
<td>7.34 ± 0.08</td>
</tr>
<tr>
<td>pEC₅₀</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>I.A.</td>
<td>(5)</td>
<td>(8)</td>
<td>(12)</td>
</tr>
<tr>
<td>AcetamidoDITMQ</td>
<td>8.96 ± 0.04</td>
<td>9.22 ± 0.07</td>
<td>8.68 ± 0.12</td>
</tr>
<tr>
<td>pEC₅₀</td>
<td>0.93 ± 0.04</td>
<td>0.84 ± 0.02</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>I.A.</td>
<td>(4)</td>
<td>(4)</td>
<td>(7)</td>
</tr>
<tr>
<td>ChloroacetamidoDITMQ</td>
<td>8.94 ± 0.07</td>
<td>8.90 ± 0.05</td>
<td>8.08 ± 0.03</td>
</tr>
<tr>
<td>pEC₅₀</td>
<td>0.81 ± 0.05</td>
<td>0.83 ± 0.02</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>I.A.</td>
<td>(4)</td>
<td>(4)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Table 2. Agonist activity of (-)-isoproterenol, acetamidoDITMQ and chloroacetamido-DITMQ on β-adrenoceptors in isolated rat tissues. Data are calculated as pEC₅₀ (-log EC₅₀, concentration required to produce a response equal to 50% of maximal response elicited by the drug) and I.A. (Intrinsic activity, maximal drug-induced response relative to the maximal response elicited by (-)-isoproterenol). The values are mean ± SEM of the number of experiments indicated in parentheses.
Figure 18. Duration of the chronotropic response after washout in rat right atria treated with (-)-isoproterenol (□), acetamidoDITMQ (●) and chloroacetamidoDITMQ (◇). The data are mean ± SEM of 3-6 experiments.
Figure 19. Concentration-dependent contraction of rat aortic tissue by phenylephrine with and without tissue preincubation (30 min) with TMQ analogs. Data are expressed as the mean ± SEM of 3-4 experiments. Panel A: Controls - first (□) and second (■) curves. Panel B: Before (◇) and after (◆) incubation with $10^{-6}$ M acetamidoDITMQ. Panel C: Before (○) and after (●) incubation with $10^{-6}$ M chloroacetamidoDITMQ.
CHAPTER 3

PHARMACOLOGICAL STUDIES ON HUMAN β-AR SUBTYPES

3.1 Aims

Pharmacological studies with selected TMQ analogs were conducted on human β-AR subtypes expressed individually in CHO cells to determine their binding affinities and biochemical potencies as agonists in terms of cAMP accumulation, in order to: (1) evaluate their β-AR subtype specificities, and (2) to evaluate species-related differences (i.e. rat versus human), if any, in pharmacological properties of the compounds on the β3-AR. To further elucidate the specificity of interactions of the compounds with the β-AR, competitive inhibition in the presence of propranolol was demonstrated in CHO cells expressing the hu-β2- and hu-β3-ARs. More detailed studies with propranolol (pA₂ determinations) were conducted with selected analogs on the hu-β2-AR in CHO cells.
3.2 Materials and Methods

3.2.1. Chemicals

The chemicals used in the study, and their sources were: Trizma-HCl, (-)-isoproterenol bitartrate, 3-isobutyl-1-methylxanthine, dimethyl sulfoxide (DMSO), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and forskolin (Sigma Chemical Co., St. Louis, MO); L-ascorbic acid (J.T. Baker Chemical Co., Phillipsburg, NJ); (+)-propranolol (Ayerst Laboratories Inc., New York, NY); [125I]-(−)-3-iodocyanopindolol (2000 Ci/mmol) (Amersham, Arlington Heights, IL); Ham's F-12 nutrient medium, Hank's phosphate buffered saline, L-glutamine, penicillin-streptomycin solution, geneticin (G418), trypsin-EDTA solution and heat inactivated fetal bovine serum (Gibco, Gaithersburg, MD). BRL 37344 was a gift from Smith Kline Beecham Pharmaceuticals (Herts, U.K.). TMQ and its analogs as shown in figure 6 and described in Appendix A, were provided by Dr. Duane Miller (Department of Pharmaceutical Sciences, University of Tennessee, Memphis, TN). All other materials used were of reagent grade. All drugs were dissolved at their highest concentration in double distilled water, unless mentioned otherwise. All subsequent dilutions were made in double distilled water for cAMP accumulation studies, and in Tris-EDTA buffer for the radioligand binding studies. Tris-EDTA (tris) buffer of following composition was used for the radioligand binding assays: 50 mM Tris, 150 mM NaCl and 20 mM EDTA, pH 7.4 in double distilled water.
3.2.2 CHO Cells: Sources and Culture Conditions.

CHO cells expressing the human β₁- and β₂-AR were obtained from Dr. L.J. Emorine (Institut Cochin de Genetique Moleculaire, Paris, France). Culture medium for these cells consisted of modified Ham's F-12 nutrient medium lacking glycine, hypoxanthine and thymidine (these were selection markers for the CHO cells expressing the β₁- and β₂-ARs), and supplemented with 10% fetal calf serum, 2mM L-glutamine and 50 U/ml-50 μg/ml penicillin-streptomycin.

CHO cells expressing the human β₃-AR (truncated β₃-AR, 402 amino acids) were obtained from Dr. Stephen Liggett, Department of Pulmonary and Critical Care Medicine, University of Cincinnati, OH). These cells were grown in culture medium consisting of Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum, 2mM L-glutamine and 50 U/ml-50 μg/ml penicillin-streptomycin. Geneticin (G418) was added to the media at a final concentration of 700 μg/ml every third passage to maintain selective growth of human β₃-AR expressing cells.

The protocol followed for culturing and harvesting cells was identical to that described in the methods section (2.2.2) of chapter 2.

3.2.3 Competitive Radioligand Binding Assays

Competitive radioligand binding assays were performed with whole CHO cells expressing the human β-AR subtypes, the assay protocol being identical to that described in chapter 2, section 2.2.3. The following concentrations of the radioligand [¹²⁵I]Iodocyanopindolol ([¹²⁵I]ICYP) were used in the assays
with the different β-AR subtypes: 30-60 pM for human β₁-AR (Kᵢ = 17 pM, Tate et al., 1991), 30-230 pM for human β₂-AR (Kᵢ = 31 pM Tate et al., 1991) and 0.20-0.30 pM for human β₃-AR (Liggett, 1992). Studies with human β₁-AR and with human β₂-AR were carried out with 12-20 x 10³ cells/250 µl total volume, whereas 40-50 x 10⁴ cells/250 µl were used in studies with human β₃-AR. Nonspecific binding was determined in the presence of (±) propranolol at 2 x 10⁻⁶M for hu-β₁- and hu-β₂-AR, and at 10⁻⁴M for hu-β₃-AR studies.

3.2.4 cAMP Accumulation Studies by Radioimmunoassay

CHO cells expressing the various subtypes of human β-AR were grown to confluency in 60 x 15 mm sterile dishes. The protocol for drug treatment and radioimmunoassay has been described previously in chapter 2, section 2.2.4. Untreated (basal) and 25 µM forskolin-treated cells were used to determine functional viability (ability to produce an increase in cAMP subsequent to drug treatment). Maximal response to isoproterenol was used as the reference (100% effect) within each experiment.

3.2.5 Inhibition of cAMP Accumulation by Propranolol

Concentration-response curves of (-)-isoproterenol, acetamidodITMQ and chloroacetamidodITMQ in the absence and presence of increasing concentrations of propranolol, were generated in CHO cells expressing the human β₂-AR. The response measured was agonist-mediated cAMP accumulation in the cells. For this purpose, the cells were grown to
confluency in 60 x 15 mm culture dishes. These were rinsed with regular Hank's buffer, followed by 30 min incubation at 37°C with modified Hank's buffer (refer chapter 2, section 2.2.4 for composition) containing 0, 0.1, 1, 10 or 100 nM propranolol. Various concentrations of the agonists were then added to the cells and incubated at 37°C for further 20 min. The buffer was then aspirated and accumulated cAMP was extracted with 2 ml of 6% trichloroacetic acid. The samples were subjected to further treatment and radioimmunoassay as per protocol described previously.

Propranolol (100 nM) inhibition of cAMP accumulations produced by the bromoacetamidoDITMQ, azidolTMQ and isothiocyanatolTMQ, in CHO cells expressing human ß-ß-AR, and that by 10 µM propranolol in CHO cells expressing human ß-AR, were measured at fixed concentrations of the agonists, by the same procedure.

3.2.6 Data Analysis

Analyses of the radioligand binding data as well as concentration versus cAMP accumulation data, were carried out using GraphPAD Inplot version 3.14 (GraphPAD Software Inc., San Diego, CA). Determination of binding affinity constants (pK,) from competition curves and of biochemical potencies (pK act) as well as maximal responses (E max) from concentration-response curves has been described earlier in chapter 2. Percentage receptor occupancy of the various ligands and relative efficacies of the agonists were also calculated using the equations described in the chapter.
Data gathered from competitive-inhibition of agonist concentration-response curves by propranolol were analyzed by the method of Arunlakshana and Schild (1959). Dose ratios (DR) were calculated for the compounds as the ratio of EC$_{50}$ of the agonist in the presence of the antagonist to that in the absence of the antagonist. The pA$_2$ value (i.e. the negative log molar concentration of antagonist that produces a two-fold shift to the right in agonist concentration-response curve) of propranolol was determined from the x-intercept of the linear regression plot of log (DR-1) versus log molar propranolol concentration, using GraphPAD version 3.4.

The unpaired Student's t-test was used to determine statistical difference in values between two compounds at a significance level of 5%.

3.3 Results

3.3.1. Binding Affinities for Human β-AR Subtypes in CHO Cells.

The non-selective β-AR agonist, (-)-isoproterenol, and 1-benzyl ring-substituted derivatives of TMQ competed for specific-bound $[^{125}I]$ICYP from the hu-β-ARs in CHO cells in a concentration-dependent manner. Additionally, the β$_3$-AR selective agonist, BRL 37344 also competitively inhibited the ICYP binding to hu-β$_3$-AR in CHO cells. The concentration-dependent competition of ICYP-binding to the hu-β-AR subtypes by these ligands are shown in figures 20, 22 and 24. The inhibitory constants (pK$_i$) of these ligands for each human receptor subtype were determined from the competition curves and are listed in Tables III, IV and V. In studies with hu-β$_1$- and hu-β$_2$-ARs, the non-specific
[125I]ICYP binding was less than 5%, whereas with hu-β3-AR subtype studies, non-specific binding ranged from 25-30% of the total binding. At high concentrations, these ligands completely inhibited specific binding of ICYP to the hu-β1- and hu-β2-ARs, whereas in the hu-β3-AR system, only 60-90% of the specific-binding of ICYP was inhibited by most TMQ analogs, an exception being azidolTMQ which completely abolished ICYP binding to this receptor subtype at high concentrations.

In general, the compounds including (-)-isoproterenol, exhibited similar binding affinities for both hu-β1- and hu-β2-ARs. However, the TMQ derivatives possessed significantly higher (10-220 fold) binding affinities for the β-ARs as compared to (-)-isoproterenol. Besides, some significant differences were found in the rank order of affinities of the TMQ analogs for the two receptor subtypes. In the hu-β1-AR system, the compounds exhibited following rank order of binding affinities (pK\textsubscript{i} in parentheses): chloroacetamidoDITMQ (8.31) > bromoacetamidoDITMQ (8.23) > isothiocyanatoTMQ (7.64) > azidolTMQ (7.45) > acetamidoDITMQ (7.32) > (-)-isoproterenol (5.97); whereas in the hu-β2-AR system, the rank order of binding affinities were bromoacetamidoDITMQ (8.48) > chloroacetamidoDITMQ (8.37) > acetamidoDITMQ (8.22) > isothiocyanatoTMQ (7.35) > azidolTMQ (7.17) > (-)-isoproterenol (6.17). The most striking difference was the nearly 10-fold lower affinity of the acetamido derivative for hu-β1-AR compared to hu-β2-AR.

Predictably, all the ligands exhibited significantly lower affinities for the hu-β3-AR compared to the other two subtypes. Typically, the compounds showed 20-50 fold lower affinities for the hu-β3-AR subtype, with the
exception of the azido derivative which exhibited only 3-6 fold difference in binding affinities for the β-AR subtypes. The acetamido derivative had a 40-fold lower affinity for the hu-β<sub>3</sub>-AR when compared to hu-β<sub>2</sub>-AR, but only 5-fold lower when compared to hu-β<sub>1</sub>-AR. The comparative affinities of the compounds for hu-β<sub>3</sub>-AR in decreasing rank order were: bromoacetamido-DITMQ (6.94) ≥ chloroacetamidoDITMQ (6.77) ≥ azidolTMQ (6.70) ≥ acetamidoDITMQ (6.63) > BRL 37344 (5.79) ≥ isothiocyanatoTMQ (5.60) > (-)-isoproterenol (4.55). Thus, all the TMQ analogs possessed binding affinities for hu-β<sub>3</sub>-AR which were equal to, or greater than those of the standard agonists, (-)-isoproterenol and BRL 37344.

3.3.2 Biochemical Activities on Human β-AR Subtypes in CHO Cells

The biochemical activities of the TMQ analogs in CHO cells expressing the three different subtypes of β-AR were determined by quantitation of agonist-stimulated cAMP accumulation in the cells, using (-)-isoproterenol as the reference agonist. In hu-β<sub>3</sub>-AR studies, the selective agonist, BRL 37344, was also included for comparative purposes. Forskolin (25μM)-stimulated levels of cAMP were determined to evaluate functional viability of the human β-ARs in the CHO cells. The basal levels, the forskolin-stimulated, and the maximal (-)-isoproterenol stimulated levels of cAMP (mean ± SEM) in CHO cells expressing the hu-β<sub>1</sub>-AR were 370 ± 68, 4748 ± 406 and 1728 ± 82 pmol/mg protein respectively; in those expressing the hu-β<sub>2</sub>-AR these were 386 ± 46, 11256 ± 605 and 3322 ± 145 pmol/mg protein respectively; and in cells expressing the hu-β<sub>3</sub>-AR the respective levels were 126 ± 23, 10573
± 924 and 3603 ± 364 pmol/mg protein. Thus, despite some differences in absolute levels of cAMP stimulation in the CHO cells expressing the different β-AR subtypes, the proportional levels of cAMP accumulation by maximal (-)-isoproterenol-stimulation compared to those by forskolin, were more or less similar (30% - 36%). This indicated a constant proportion of adenylyl cyclase was stimulated via agonist-activated β-AR in the three systems, and therefore, comparisons of biochemical activities of the various agonists studied could be made upon normalization of the data.

Tables III, IV and V summarize the biochemical potencies as pK_{act} (negative log molar agonist-concentration eliciting a half-maximal increase in cAMP accumulation) and maximal activity as E_{max} (maximal cAMP accumulation produced by the agonist relative to that of (-)-isoproterenol) of the TMQ analogs and standard compounds in the three human β-AR systems. Figures 21, 23 and 25 illustrate β-AR subtype mediated cAMP accumulation levels as a function of agonist concentration (panel A) and as a function of receptor occupancy (panel B).

The rank order of biochemical potencies of the compounds in CHO cells expressing the hu-β_{1}-AR were (pK_{act} in parentheses): azidolTMQ (10.39) ≥ bromoacetamidoDITMQ (10.13) ≥ chloroacetamidoDITMQ (9.86) > acetamidoDITMQ (9.49) ≥ isothiocyanatoTMQ (9.42) > (-)-isoproterenol (8.93). Thus, all the TMQ analogs were significantly more potent than (-)-isoproterenol, ranging from a minimum of 3-fold, to a maximum of 29-fold greater potency. Also, the maximal activities of the TMQ analogs were 1.13 - 1.34 times higher than that of (-)-isoproterenol, indicating these compounds
to be full agonists in the hu-β₁-AR system. With the exception of the azido derivative, all the TMQ derivatives occupied larger fractions of the receptor for response equivalent to 50% maximal response by (-)-isoproterenol. For this response, (-)-isoproterenol occupied 0.155% of total receptors, whereas the azidolTMQ required to occupy only 0.063% of the receptors. The efficacies of the compounds in decreasing rank order were: azidolTMQ, 2.45 (0.155/0.063) > (-)-isoproterenol, 1.00 > acetamidoDITMQ, 0.37 (0.155/0.42) > bromoacetamidoDITMQ, 0.16 (0.155/0.98) > isothiocyanatolTMQ, 0.15 (0.155/1.03) > chloroacetamidoDITMQ, 0.073 (0.155/2.12).

In CHO cells expressing the hu-β₂-AR, the compounds exhibited following rank order of potencies (pKₐ in parentheses): azidolTMQ (10.88) > acetamidoDITMQ (10.83) > bromoacetamidoDITMQ (10.54) > chloroacetamidoDITMQ (9.88) > isothiocyanatolTMQ (9.29) > (-)-isoproterenol (8.41). Thus, the TMQ analogs possessed significantly greater potencies at the hu-β₂-AR compared to (-)-isoproterenol. The maximal activities of TMQ analogs were comparable to that of (-)-isoproterenol. The efficacies of the TMQ derivatives relative to (-)-isoproterenol were calculated from the hu-β₂-AR occupancy plots, and in decreasing rank order were: azidolTMQ, 36.7 (0.755/0.021) > acetamidoDITMQ, 2.42 (0.755/0.31) > isothiocyanatolTMQ, 1.11 (0.755/0.68) > (-)-isoproterenol, 1.00 (0.755/0.755) > bromoacetamidoDITMQ, 0.96 (0.755/0.79) > chloroacetamidoDITMQ, 0.19 (0.755/4.00).

In the hu-β₃-AR expressing CHO cells, the TMQ analogs exhibited greater biochemical potencies when compared to those of (-)-isoproterenol.
and BRL 37344, and the rank order of potencies were (pK<sub>act</sub> in parentheses):

- azidolTMQ (10.32) > bromoacetamidoDITMQ (9.35) ≥ acetamidoDITMQ (8.96) ≥ chloroacetamidoDITMQ (8.68) > isothiocyanatoTMQ (8.02) ≥ (-)-isoproterenol (7.99) ≥ BRL 37344 (7.64). BRL 37344 was a partial agonist with maximal activity (mean ± SEM) of 65 ± 4.2% that of (-)-isoproterenol mediated maximal activity, whereas the TMQ analogs were full agonists, with <i>E<sub>max</sub></i> values ranging from 101 ± 8.3% to 114 ± 7.4% of maximal (-)-isoproterenol mediated activity. At the hu-β<sub>3</sub>-AR, the azido, bromoacetamido, acetamido and chloroacetamido derivatives were 214-, 23-, 9- and 5-fold more potent compared to (-)-isoproterenol, and 479-, 51-, 21- and 11-fold more potent than BRL 37344. In terms of % hu-β<sub>3</sub>-AR occupancy, the TMQ analogs had relatively lower efficacy as compared to (-)-isoproterenol, but possessed significantly greater efficacy than BRL 37344. The relative efficacies in this system were as follows: (-)-isoproterenol, 1.00 (0.027/0.027) > azidolTMQ, 0.69 (0.027/0.040) > isothiocyanatoTMQ, 0.13 (0.027/0.21) > bromoacetamidoDITMQ, 0.078 (0.027/0.35) > acetamidoDITMQ, 0.036 (0.027/0.76) ≥ chloroacetamidoDITMQ, 0.032 (0.027/0.86) > BRL 37344, 0.0041 (0.027/6.64).

### 3.3.3 Human β-AR Subtype Selectivity

In general, the TMQ compounds exhibited similar binding affinities and biochemical potencies in hu-β<sub>1</sub>- and hu-β<sub>2</sub>-AR subtypes. The acetamido analog was the only compound which was functionally more potent (22-fold) at the hu-β<sub>2</sub> as compared to hu-β<sub>1</sub>-AR.
Comparing the binding affinities of the compounds for the hu-β₁- and hu-β₂-AR with those for the hu-β₃-AR, (-)-isoproterenol was 26- and 42-fold more potent, respectively, whereas the chloroacetamido and bromoacetamido analogs of DITMQ were 35- and 40-fold, and 19- and 35-fold more potent at these two receptor subtypes, respectively. While the acetamido and azido analogs had similar affinities for the three receptors, the isothiocyanato compound exhibited significantly lower affinity for the hu-β₃-AR in comparison to the other two subtypes, being 110- and 56-fold lower for the hu-β₃-AR as compared to those at the hu-β₁- and hu-β₂-AR, respectively.

Functionally, (-)-isoproterenol, as well as bromoacetamidoDITMQ and azidoDITMQ were non-selective for the β-AR subtypes. The chloroacetamido and isothiocyanato compounds showed similar potencies for the hu-β₁- and hu-β₂-ARs; however these were only 16-fold and 20-fold greater respectively, when compared to their potencies at the hu-β₃-AR. The acetamido analog exhibited some selectivity for the hu-β₂-AR, being 22- and 74-fold more potent at this subtype compared to those at the hu-β₁- and hu-β₃-AR, respectively.

3.3.4 Species-Related Differences in Pharmacological Properties

When the binding affinities and biochemical activities of the compounds were compared between the rat and human β₃-AR, (-)-isoproterenol and the TMQ analogs in general, did not exhibit any significant differences in the potencies. The functional potencies of the TMQ analogs were slightly higher at the rat β₃-AR, but the maximal activities were higher at the hu-β₃-AR. The azido derivative was not studied at the rat β₃-AR. The most significant
difference related to these species was seen with BRL 37344. Compared to
the rat receptor subtype, BRL 37344 possessed a 15-fold lower binding
affinity and an 18-fold lower biochemical potency at the hu-β3-AR. In contrast
to its full agonist activity on the rat β3-AR, BRL 37344 was a partial agonist
at the human receptor subtype.

3.3.5 Propranolol Inhibition of Agonist-induced cAMP Accumulation in CHO
Cells Expressing Human β2- and β3-ARs.

TMQ analog-mediated cAMP accumulations in CHO cells expressing the
hu-β2- and hu-β3-AR were determined in the absence and presence of
propranolol at concentrations of 100 nM and 10 μM respectively. The
concentration-dependent inhibition of (-)-isoproterenol, acetamidoDITMQ and
chloroacetamidoDITMQ activities on hu-β2-AR was studied in greater detail,
and results are described in the following section. Figure 26 demonstrates
that propranolol (100 nM) inhibited cAMP accumulation by different
concentrations of the bromoacetamido (A), isothiocyanato (B) and azido (C)
derivatives of TMQ at hu-β2-AR. Propranolol treatment resulted in lowering
of cAMP levels to below the basal levels in these cells. Concentration-
dependent accumulation of cAMP by various agonists in cells expressing the
hu-β3-AR in the absence and presence of 10 μM propranolol is demonstrated
in figure 27 (A-F). In this system, propranolol produced significant reductions
in agonist-mediated cAMP levels, and these inhibitory effects were
centration-dependent.
3.3.6 Schild Regression Analysis of Propranolol Inhibition of Agonist-induced cAMP Accumulations in CHO Cells Expressing Human β2-AR.

The concentration-dependent biochemical activities of (-)-isoproterenol, acetamidoDITMQ and chloroacetamidoDITMQ were studied in the absence and in the presence of different concentrations of propranolol. The concentration-response curves of the various agonists were shifted to the right progressively by increasing concentrations of the antagonist, as shown in figure 28 (A-C), indicating competitive inhibition by propranolol. However, the concentration-response curves of the agonists were differentially affected by antagonist concentrations. While (-)-isoproterenol activity was more or less unaffected in the presence of 0.1 nM and 1 nM propranolol, significant rightward shifts in the curves were observed for the two TMQ derivatives. The EC50 values determined from these curves are listed in Table 6. Schild regression analyses of agonist dose-ratios as a function of antagonist concentration, yielded straight lines for each agonist compound with slope not significantly different from 1. However, each line intersected with the abscissa at different points, thereby giving rise to different pA2 values of propranolol for each agonist (figure 29). Dose-ratio plots of (-)-isoproterenol yielded a regression line with slope of 1.31 ± 0.24, and a pA2 of 8.24 (5.75 nM) which was in close agreement with previously determined value of 5 nM (Wilson et al., 1984). The slopes of linear regression plots of acetamido and chloroacetamido TMQ analogs were 0.92 ± 0.04 and 0.89 ± 0.06, respectively, and the corresponding pA2 values were 11.44 (3.63 pM) and
10.30 (50.12 pM), respectively. The correlation coefficients of the regression plots ranged from 0.984 to 0.999.

3.4. Discussion

Catecholamine agonists and closely related analogs have exhibited nanomolar potencies for stimulating adenylyl cyclase in CHO cells expressing the human β-ARs (Tate et al., 1991; Strosberg, 1995; Pietri-Rouzel et al., 1995). The TMQ analogs evaluated in these studies possessed comparable agonist potencies on these β-ARs, and these were higher compared to the potencies of the standard agonists, isoproterenol and BRL 37344. Most surprisingly, the azido derivative was found to be the most potent agonist on all the β-AR subtypes, including β3-AR, with EC50 values in the picomolar range. The higher potencies of most of these derivatives on β1- and β2-ARs as compared to the β3-AR were expected due to the presence of catechol group on the molecule. The somewhat higher selectivity for the β1/β2-ARs over β3-AR was also observed previously in functional studies on rat tissues containing these receptors (Konkar et al., 1996; Fraundorfer et al., 1994). While replacement of the catechol hydroxyls of TMQ with 6,7-dimethoxy or a 6,7-methylenedioxy groups had resulted in significant abrogation of binding affinities and agonist activities, the replacement of a hydroxyl with chlorine at the 7-position of TMQ, or bioisosteric replacement of the dihydroxytetrahydroisoquinoline ring of TMQ with tetrahydrothiazolopyridine has yielded compounds with partial agonist activities on the β3-AR and minimal agonist activities on the other two subtypes (Konkar et al., 1997). Moreover, the
diiodo analog of the bioisosteric compound exhibited greater potency and selectivity for the human \( \beta_3 \)-AR. Based on the current studies, it is postulated that further modifications (like acetamido or azido) on the 4'-position of the benzyl ring of the bioisosteric analog of DITMQ will yield compounds with very high potencies and selective agonist activities on the \( \beta_3 \)-AR.

Generally, the \( \beta_3 \)-AR agonists being developed for therapeutic purposes are first tested in rodent models. Cloning of the \( \beta_3 \)-ARs in CHO cells has simplified the development process, since initial characterization of new compounds can now be carried out in these recombinant systems with relative ease and convenience. Pharmacological properties of the rat \( \beta_3 \)-AR in CHO cells have been reported to be similar to the properties exhibited by the atypical receptors in rat adipocytes (Granneman et al., 1991; Muzzin et al., 1991). Compounds yielding promising results in the rat model have also been investigated on the cloned human \( \beta_3 \)-AR. However, differences in agonist properties have been reported for several compounds between the rat and human \( \beta_3 \)-AR. Rank orders of agonist potencies and relative intrinsic activities of several agonists were found to be different for the rat and human \( \beta_3 \)-ARs expressed in CHO cells (Liggett, 1992). Most notably, BRL 37344, a potent and full agonist on the rat \( \beta_3 \)-AR, demonstrated partial agonist activity on the human \( \beta_3 \)-AR, even though the endogenous catecholamines exhibited similar properties in the two systems (Granneman et al., 1991; Liggett, 1992). In our studies too, BRL 37344 exhibited remarkable differences in the binding affinities, agonist potencies and relative intrinsic activities on the rat and human \( \beta_3 \)-ARs. On the other hand, the pharmacological properties of the TMQ
analogs were more or less similar on the two receptors, and these compounds were full agonists on the β₂-ARs of both species. Although the reason for the species difference observed with BRL 37344 is not yet elucidated, the results indicate there might be critical differences in ligand interactions with receptors of the two species that may account for differential activation of G-protein coupling, and stimulation of adenylyl cyclase activity.

A concentration-dependent inhibition of TMQ analog-induced cAMP accumulation by propranolol was observed on human β₂- and β₃-ARs expressed in CHO cells, and it is not unreasonable to assume that similar results would be observed on human β₁-AR and rat β₃-AR. Propranolol is a competitive reversible antagonist on the β-ARs, and nonselective on β₁- and β₂-ARs. According to drug-receptor theory, when the agonist and antagonist bind reversibly to the same recognition sites on the receptor and compete for occupation of such sites, competitive reversible antagonism results (Kenakin, 1993). Such antagonism is surmountable by increasing concentrations of the agonist. Competitive reversible antagonists influence the concentration-response curves of agonists by producing a progressive, rightward shift in the curve in the presence of increasing concentrations without affecting the maximal response of the agonist. This pattern was observed for the concentration-response curves of isoproterenol and the selected TMQ analogs on human β₂-AR in the presence of increasing concentrations of propranolol, indicating a common site of reversible interactions on this receptor. Schild regression plots of the concentration-response curves of selected agonists in the absence and presence of increasing concentrations of propranolol have
been used as a powerful tool for determining the dissociation constant of the antagonist ($K_a$) and more importantly, for receptor classification (Arunlakshana and Schild, 1959). The latter is based on the assumption that target sites on the receptor for agonists are more or less homogeneous since they would have to respond to the same transducing elements, whereas antagonists need not fit into the target site, but rather only need to occlude it. This could result in numerous possible binding sites for antagonists on a receptor that could vary with the composition and orientation of the receptor protein on the cell membrane. Thus, any difference in the dissociation constants of an antagonist in two systems would be indicative of different receptor populations, irrespective of the agonist used.

Propranolol inhibition studies on human $\beta_2$-AR of concentration-response curves of (-)-isoproterenol, acetamidoDITMQ and chloroacetamidoDITMQ were indicative of competitive reversible antagonism. However, different $pA_2$ ($pK_a$) values for the antagonist were obtained upon use of these three agonists. Since a homogeneous receptor system was used in these studies, deviation from the expected results can only be explained by differences in sites of interaction of the different agonists with the $\beta$-AR. In comparison with catecholamine agonists such as isoproterenol, the TMQ analogs differ in several respects. For example, TMQ and its analogs lack the presence of a $\beta$-hydroxyl group, have a semirigid tetrahydroisoquinoline nucleus, and possess a large lipophilic 1-benzyl substituent which is analogous to an N-substituent on catecholamines (Feller and Miller, 1978; Fraundorfer, 1993). Therefore, the N-substituent groups of (-)-isoproterenol (a small lipophilic
isopropyl group) and the TMQ analogs (a large lipophilic benzyl ring) will have
different sites of interaction with transmembrane domains of the β-AR. Accordingly, if this interaction is important for the agonist properties of the
TMQ compounds, the degree of competition for these sites by propranolol
would be different. The higher pA₂ values of 11.40 and 10.30 for the
acetamido- and chloroacetamido-DITMQ analogs compared to 8.24 for (-)-
isoproterenol indicate that propranolol is considerably more potent (about
1400- and 70-fold, respectively) as an inhibitor of the effects of the TMQ
analogs than that of (-)-isoproterenol. In other words, propranolol shares more
common sites on the receptor with the TMQ analogs than with (-)-
isoproterenol.

It is known that (-)-isoproterenol interacts predominantly with amino
acids on tm3, 4, 5 and 6 of the β-AR, whereas typical antagonists such as
propranolol are proposed to interact additionally with amino acids on tm1, 2,
6 and 7 (Hockerman et al., 1996). Accordingly, we propose that there may
be an overlap of a binding site for propranolol with the postulated
"hydrophobic" binding site of the 1-benzyl side chain of the TMQ analogs.
Thus, the existence this common site of interaction of TMQ analogs and
propranolol would explain the different potencies of propranolol against the
TMQ analogs versus (-)-isoproterenol. The log unit difference between pA₂
values of propranolol for the acetamido and chloroacetamido analogs of
DITMQ further emphasize some differences in the nature and degree of
interactions of the reversible (acetamido) and irreversible (chloroacetamido)
analogs of TMQ with the β-AR. Therefore, affinity binding studies were
carried out on human $\beta_2$-AR with selected TMQ analogs, and these are described in the following chapter.
Figure 20. Concentration-dependent inhibition of specific \([^{125}\text{I}]\text{ICYP}\) binding to hu-\(\beta_1\)-AR in CHO cells by selected \(\beta\)-AR ligands. The data are mean ± SEM of \(n = 5\)-7 experiments. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamidoDITMQ (○); bromoacetamidoDITMQ (▲); isothiocyanatoTMQ (■); azidoTMQ (○).
Figure 21. Concentration-dependent increases in cAMP accumulation by selected compounds in CHO cells expressing the hu-β₁-AR as a function of concentration (panel A) and as a function of receptor occupancy (panel B). Data are mean ± SEM of n = 3-6 experiments. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamido-DITMQ (○); bromoacetamidoDITMQ (●); isothiocyanatoDITMQ (■); azidoDITMQ (○).
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<th>Compound</th>
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<th>$pK_{act}$</th>
<th>% $E_{max}$</th>
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<tr>
<td>(-)-Isoproterenol</td>
<td>5.97 ± 0.19 (7)</td>
<td>8.93 ± 0.14 (3)</td>
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<td>AcetamidoDITMQ</td>
<td>7.32 ± 0.07 (5)</td>
<td>9.49 ± 0.15 (5)</td>
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<td>ChloroacetamidoDITMQ</td>
<td>8.31 ± 0.10 (7)</td>
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<td>BromoacetamidoDITMQ</td>
<td>8.23 ± 0.06 (5)</td>
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<td>108 ± 3.1</td>
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<tr>
<td>IsothiocyanatolTMQ</td>
<td>7.64 ± 0.04 (5)</td>
<td>9.42 ± 0.07 (6)</td>
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<td>AzidoTMQ</td>
<td>7.45 ± 0.10 (6)</td>
<td>10.39 ± 0.18 (6)</td>
<td>134 ± 10.6</td>
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Table 3. Inhibition constants ($-\log K_i$ or $pK_i$) for binding and functional activity constants ($-\log EC_{50}$ or $pK_{act}$) for cAMP accumulation in CHO cells expressing the hu-$\beta_1$-AR. $E_{max}$ is the maximal cAMP accumulation stimulated by the compounds relative to that by (-)-isoproterenol. Values are mean ± SEM of the number of replicate experiments indicated in parentheses.
Figure 22. Concentration-dependent inhibition of specific [125I]ICYP binding to hu-β2-AR in CHO cells by selected β-AR ligands. Data are mean ± SEM of n = 6-7 experiments. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamidoDITMQ (◇); bromoacetamidoDITMQ (♦); isothiocyanatoDITMQ (■); azidolTMQ (○).
Figure 23. Concentration-dependent increases in cAMP accumulation by selected compounds in CHO cells expressing the hu-β₂-AR as a function of concentration (panel A) and as a function of receptor occupancy (panel B). Data are mean ± SEM of n = 4-10 experiments. Key. (-)-isoproterenol (○); acetamidoDITMQ (●); chloroacetamido-DITMQ (○); bromoacetamidoDITMQ (●); isothiocyanatoDITMQ (■); azidoDITMQ (○).
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<th>Compound</th>
<th>$pK_i$</th>
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<th>% $E_{max}$</th>
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<tr>
<td>(-)-Isoproterenol</td>
<td>$6.17 \pm 0.12$ (6)</td>
<td>$8.41 \pm 0.17$ (10)</td>
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<td>AcetamidoDITMQ</td>
<td>$8.22 \pm 0.08$ (6)</td>
<td>$10.83 \pm 0.12$ (10)</td>
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<td>ChloroacetamidoDITMQ</td>
<td>$8.37 \pm 0.18$ (6)</td>
<td>$9.88 \pm 0.18$ (9)</td>
<td>$94 \pm 3.4$</td>
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<td>BromoacetamidoDITMQ</td>
<td>$8.48 \pm 0.14$ (7)</td>
<td>$10.54 \pm 0.12$ (5)</td>
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<td>IsothiocyanatoTMQ</td>
<td>$7.35 \pm 0.03$ (7)</td>
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<td>AzidoTMQ</td>
<td>$7.17 \pm 0.05$ (6)</td>
<td>$10.88 \pm 0.08$ (4)</td>
<td>$97 \pm 6.0$</td>
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Table 4. Inhibition constants ($-log K_i$ or $pK_i$) for binding and functional activity constants ($-log EC_{50}$ or $pK_{act}$) for cAMP accumulation in CHO cells expressing the hu-B2-AR. $E_{max}$ is the maximal cAMP accumulation stimulated by the compounds relative to that by (-)-isoproterenol. Values are mean $\pm$ SEM of the number of replicate experiments indicated in parentheses.
Figure 24. Concentration-dependent inhibition of specific [\(^{125}\text{I}\)]ICYP binding to hu-\(\beta\)_2-AR in CHO cells by selected \(\beta\)-AR ligands. Data are mean ± SEM of \(n = 4-6\) experiments. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamidoDITMQ (○); isothiocyanatoDITMQ (■); azidoDITMQ (○); BRL37344 (∗).
Figure 25. Concentration-dependent increases in cAMP accumulation by selected compounds in CHO cells expressing the hu-β3-AR as a function of concentration (panel A) and as a function of receptor occupancy (panel B). Data are mean ± SEM of n = 4-10 experiments. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamido-DITMQ (○); bromoacetamidoDITMQ (♦); isothiocyanatoDITMQ (■); azidolTMQ (○); BRL 37344 (★).
Table 5. Inhibition constants (-log $K_i$ or $pK_i$) for binding and functional activity constants (-log $EC_{50}$ or $pK_{act}$) for cAMP accumulation in CHO cells expressing the hu-$\beta_2$-AR. $E_{max}$ is the maximal cAMP accumulation stimulated by the compounds relative to that by (-)-isoproterenol. Values are mean ± SEM of the number of replicate experiments indicated in parentheses.

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<tr>
<th>Compound</th>
<th>$pK_i$</th>
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<th>% $E_{max}$</th>
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<td>(-)-Isoproterenol</td>
<td>4.55 ± 0.14 (6)</td>
<td>7.99 ± 0.23 (4)</td>
<td>100</td>
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<tr>
<td>AcetamidoDITMQ</td>
<td>6.63 ± 0.14 (5)</td>
<td>8.96 ± 0.15 (5)</td>
<td>101 ± 8.3</td>
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<td>ChloroacetamidoDITMQ</td>
<td>6.77 ± 0.13 (5)</td>
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<td>BromoacetamidoDITMQ</td>
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<td>IsothiocyanatoTMQ</td>
<td>5.60 ± 0.15 (5)</td>
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<td>114 ± 7.4</td>
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<td>AzidoTMQ</td>
<td>6.70 ± 0.07 (6)</td>
<td>10.32 ± 0.09 (5)</td>
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<td>BRL37344</td>
<td>5.79 ± 0.12 (4)</td>
<td>7.64 ± 0.19 (4)</td>
<td>65 ± 4.2</td>
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Figure 26. cAMP Accumulation in CHO cells expressing the hu-β₂-AR by increasing concentrations of agonists in the absence (diagonal bars) and in presence (checkered bars) of 100 nM propranolol. Data are mean ± SEM of three or more experiments. Panels: (A) $10^{-10}$ & $10^{-9}$ M bromoacetamido-DITMQ; (B) $10^{-10}$ & $10^{-9}$ M isothiocyanatoTMQ; (C) $10^{-10}$ & $10^{-9}$ M azidoTMQ.
Figure 26.

A

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

B

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

C

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

[Diagram showing three graphs with % Maximal (-)-Isoproterenol Stimulated cAMP Accumulation on the y-axis and varying conditions on the x-axis.]
Figure 27. Isoproterenol and TMQ analog induced cAMP accumulations in CHO cells expressing the hu-β3-AR in the absence (diagonal bars) and in presence (checkered bars) of 10 μM propranolol. Data are mean ± SEM of three or more experiments. Panels: (A) 10⁻⁷, 10⁻⁶ & 10⁻⁵ M (-)-isoproterenol. (B) 10⁻⁹ & 10⁻⁸ M acetamidoDITMQ; (C) 10⁻⁸ & 10⁻⁷ M chloroacetamidoDITMQ; (D) 10⁻⁹ & 10⁻⁸ M bromoacetamidoDITMQ; (E) 10⁸ & 10⁷ M isothiocyanatolTMQ; (F) 10⁻⁹ & 10⁻⁸ M azidolTMQ.
Figure 27.

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

(a) [Irc-o]

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

(b) [Irc-o]

% Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

(c) [Irc-o]
Figure 28. Agonist-induced concentration-dependent increases in cAMP accumulation in CHO cells expressing the hu-β2-AR in the absence (□) and presence of 0.1 nM (△), 1 nM (○), 10 nM (■) and 100 nM (⋆) propranolol. Data are mean ± SEM of n = 3-10 experiments. Panel A: (-)-isoproterenol; panel B: acetamidoDITMQ and panel C: chloroacetamidoDITMQ.
Figure 28.

(A) [Drug] (M) % Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

(B) % Maximal (-)-Isoproterenol Stimulated cAMP Accumulation

(C) % Maximal (-)-Isoproterenol Stimulated cAMP Accumulation
<table>
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<tr>
<th>[Propranolol] (nM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; Values (nM)</th>
<th>((-)-Isoproterenol)</th>
<th>AcetamidoDITMQ</th>
<th>ChloroacetamidoDITMQ</th>
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Table 6. Agonist concentrations (EC<sub>50</sub>) producing half maximal cAMP accumulation in CHO cells expressing hu-β<sub>2</sub>-AR in the absence and presence of various concentrations of propranolol. The values were obtained from concentration-response curves of 3-10 replicate experiments as shown in figure 28.
Figure 29. Schild regression analysis of propranolol inhibition of agonist-mediated cAMP accumulation in CHO cells expressing the hu-β2-AR. Ordinate: Logarithm of dose-ratio minus 1. Abscissa: Molar propranolol concentration. The dose ratios were calculated from EC$_{50}$ values given in Table VI. The pA$_2$ value of propranolol (x-intercepts), slope and correlation coefficient (r) of the linear regression plot for data with each agonist are tabulated. Key. (-)-isoproterenol (□); acetamidoDITMQ (●); chloroacetamidoDITMQ (○).
CHAPTER 4

AFFINITY AND PHOTOAFFINITY STUDIES
ON HUMAN $\beta_2$-ADRENOCEPTOR

4.1 Aims

Previous studies from our laboratory have indicated that substitutions at the 3', 4'- or 3',4',5'-positions of the 1-benzyl group of trimetoquinol (TMQ) are important in determining the potency for activation of $\beta$-adrenoceptors ($\beta$-ARs) (Konkar et al., 1996, De LosAngeles et al., 1996). Mono- or diiodo substitution on the 1-benzyl ring is associated with high functional potency on tissues containing various subtypes of $\beta_2$-ARs. In chapter 3, evidence was also presented indicating that propranolol inhibition of adenylyl cyclase activation by the 4'-acetamido- and 4'-$\alpha$-chloroacetamido-3',5'-diido- substituted TMQ analogs was significantly different from that by isoproterenol. Thus, these data suggest that TMQ analogs interact differently with the human $\beta_2$-AR, as compared to the classical agonist, isoproterenol. In addition several of the TMQ analogs possessed higher affinity for the hu-$\beta_2$-AR as compared to the other subtypes.
One objective of this research is to examine whether appropriately modified 1-benzyl TMQ analogs are suitable as affinity probes for β-AR subtypes. In order to examine the sites of interactions of the 1-benzyl group of TMQ, a series of affinity TMQ analogs were prepared which contained reactive substituents on the 4'-position of this ring. Compounds synthesized were chloroacetamidoDITMQ, bromoacetamidoDITMQ, isothiocyanatolTMQ and azidolTMQ (see Figure 1 for structures and Appendix A for chemical description). Therefore, it was desirable to characterize the affinity and photoaffinity characteristics of these compounds in CHO cells expressing the hu-β2-AR. Moreover, the significantly higher affinity of iodocyanopindolol for the hu-β2-AR, with $K_d$ in picomolar range compared to that in the nanomolar range for hu-β2-AR (see chapter 3), provided additional justification for using the former receptor system to characterize the affinity properties of these compounds.

The studies described in this chapter include (1) the determination of time- and concentration-dependent affinity binding of the haloacetamido-DITMQ, isothiocyanatolTMQ and azidolTMQ analogs to hu-β2-AR in CHO cells and (2) protection assays with competing β-AR ligands and nucleophiles to evaluate the site-specificity and chemical nature of interaction of these affinity ligands with the receptors. Photoaffinity properties of azidolTMQ on the hu-β2-AR were determined in a similar manner to those outlined for the reactive affinity labels.
4.2 Materials and Methods

4.2.1. Chemicals

The chemicals used and their sources were same as those described in the materials section of chapter 3 (see section 3.2.1). Additional substances included glutathione (reduced form) and L-cysteine, which were obtained from Sigma Chemical Company (St. Louis, MO). Tris-EDTA (tris) buffer, pH 7.4, containing 50 mM Tris, 150 mM NaCl and 20 mM EDTA, was used for the affinity and photoaffinity binding studies.

4.2.2. CHO Cells: Source and Culture Conditions

CHO cells expressing human β2-ARs used in these studies were obtained from Dr. L.J. Emorine (Institut Cochin de Genetique Moleculaire, Paris, France) and the culturing conditions were as described in chapter 3, section 3.2.2.

4.2.3. Time and Concentration Dependence of Affinity Binding

CHO Cells expressing the human β2-AR (about 30-40 x 10^3 cells/150 μl) were suspended in 1.2 ml tris buffer in microfuge tubes and incubated at room temperature in a rotating shaker (Robbins Scientific) with acetamidoDITMQ (K_i = 6.0 nM) chloroacetamidoDITMQ (K_i = 4.3 nM) or isothiocyanatolTMQ (K_i = 45 nM) at concentrations of 3-, 10-, 30- and 100-fold of the K_i values for time periods ranging from 2 min to 45 min. Incubations were stopped by centrifugation of the cell suspension at 1500 x g in microcentrifuge (Eppendorf.
Model 5415C). Cell pellets were resuspended in 1.2 ml of fresh buffer, and the samples were placed again on the shaker for about 15 min to allow for drug equilibrium (bound and free drug), followed by recentrifugation and resuspension. This washing procedure was repeated three times for each sample. Protein contents of the final reconstituted cell suspensions were determined by the method of Lowry et al. (1951).

Triplicate aliquots of normalized suspensions (20-30 x 10^3 cells/aliquot) were incubated with 60-240 pM of [125I]ICYP in a final volume of 250 μl in buffer for one hour at 37°C. Binding reactions were terminated by rapid filtration (5 mL of ice cold tris buffer x 2 times) of the samples through Whatman GF/B filters on a Brandel Model 12-R cell harvester, and the radioactivity present on filters was measured in a gamma counter (Model 1470 Wizard, Wallac Inc., Gaithersburg, MD). Non-specific binding for each sample aliquot was determined in the presence of 2 x 10^6 M (±) propranolol.

4.2.4. Protection Assays with Competing β-AR Ligands and Nucleophiles

Suspensions (1.2 ml) of CHO cells expressing the human β2-AR in tris buffer (30-40 x 10^3 cells/150 μl) were incubated for 20 min at room temperature in a rotating shaker (Robbins Scientific, 18 rpm) with either (-)-isoproterenol or propranolol at molar concentrations of 100- or 300-fold of the K_i values or acetamidoDITMQ at 30- or 100-fold of its K_i value. IsothiocyanatolTMQ was then added to the suspension and incubated for 5 min at molar concentrations of 3- or 10- fold of its K_i, respectively. The competitive binding reactions were stopped by centrifugation of the cell
suspensions at 1500 x g in microcentrifuge (Eppendorf Model 5415C), and
cells were subjected to 3 washes with buffer, incubating at room temperature
in the rotating shaker for 10-15 min between each wash. Normalization of
protein content of the reconstituted suspensions and radioligand binding
assays were carried out as described previously.

Protection assays were carried out similarly with 0.01-0.10M
glutathione or L-cysteine at pH 7.4 as protecting agents. Nucleophile
solutions (pH 7.4) were prepared in tris buffer and used on the same day.
IsothiocyanatoTMQ was allowed to interact with the nucleophiles in freshly
prepared solution for about 20 min after which cells were added and allowed
to react for 5 min. The cell suspension was then centrifuged at 1500 x g for
2 min. The washing and assay protocol was identical to that described above.

As in previous experiments, controls included treatments with the
protecting agents in the absence of affinity ligands.

4.2.5. Time and Concentration Dependent Photoaffinity Binding

CHO Cells expressing the human β2-AR (about 30-40 x 10³ cells/150 µl)
were suspended in 1.2 ml tris buffer in microfuge tubes and incubated at room
temperature under red light in a rotating shaker (Robbins Scientific, 18 rpm)
with azidolTMQ (K_i = 68 nM) at concentrations of 1-, 3-, 10- and 100-fold of
the K_i value for 1 hour. The suspensions were then transferred to quartz
tubes and exposed to UV light of 350 nm at about 10 cms distance for 30
secs or 5 min. The photolyzed suspensions were then centrifuged for 2 min
at about 1500 x g in a microcentrifuge (Eppendorf Model 5415C). Cell pellets
were resuspended in 1.2 ml of fresh buffer, and the samples were placed again on the shaker for about 15 min in the dark to allow equilibrium of the drug between bound and free forms followed by recentrifugation and resuspension. This washing procedure was repeated twice for a total of three times for each sample. Protein contents of the final reconstituted cell suspensions were determined by the method of Lowry et al. (1951).

Triplicate aliquots of normalized suspensions (20,000 to 30,000 cells/aliquot) were incubated with 60-240 pM of [125I]ICYP in a final volume of 250 μl in buffer for one hour at 37°C. Binding reactions were terminated, and radioactivity was measured as described earlier in Chapter 2. Non-specific binding for each sample aliquot was determined in the presence of 2 x 10^-6 M (±) propranolol. In control experiments, untreated cell suspensions were subjected to the identical photolysis procedure and evaluated for radioligand binding. Non-photolysed cells, in the absence of the photoaffinity ligand, were utilized for determining total binding of the radioligand.

4.2.6. Protection against Photoaffinity Binding

Suspensions (1.2 ml) of CHO cells expressing the human β2-AR in tris buffer (30-40 x 10^3 cells/150 μl) were incubated under red light for 1 hour at room temperature (25°C) in a rotating shaker (Robbins Scientific, 18 rpm) with either (-)-isoproterenol (300-Kᵢ), propranolol(300-Kᵢ), acetamidoDITMQ (100-Kᵢ) or L-cysteine (0.1 mM) along with azidoDITMQ (10-Kᵢ). The equilibrated suspensions were then transferred to quartz tubes and exposed to UV light at 350 nm for 30 sec. Following photolysis, the suspensions were centrifuged...
at 1500 x g in a microcentrifuge (Eppendorf Model 5415C) and the cells were subjected to washing, protein normalization and radioligand binding procedure described above. In control experiments, cells were equilibrated for 1 hour with the protecting agents in the absence of the azido derivative and subjected to identical photolysis, washing and radioligand binding assay procedures.

4.2.7. Data Analysis

The unpaired Student's t-test was used to determine statistical difference of means for control and treatment groups at a significance level of 5%.

4.3. Results

4.3.1. Effect of Incubation Time and Ligand Concentration on Affinity Binding

In reversible competitive radioligand binding experiments, all of the selected 1-benzyl substituted analogs of TMQ, including acetamido, α-haloacetamido and isothiocyanato derivatives, had inhibited [³⁵S]ICYP binding to the hu-β²-AR in CHO cells in a concentration-dependent manner, with binding affinities in the nanomolar ranges (see chapter 3, section 3.3.1). In washout experiments, concentration-dependent inhibition of [³⁵S]ICYP to hu-β²-AR was observed in cells pretreated with isothiocyanatoDITMQ and with chloroacetamidoDITMQ. In contrast, acetamidoDITMQ pretreatment did not significantly affect radioligand binding after washout, irrespective of
concentration used (figure 30). Depending upon the concentration used, isothiocyanatoITMQ inhibited ICYP binding by 35-85% whereas chloroacetamidoDITMQ inhibited ICYP binding by a maximum of only 30-40% of the total radioligand binding. Non-specific radioligand binding to the receptors was around 5%. Thus, isothiocyanatoITMQ was a more effective affinity ligand on the hu-β₂-AR, whereas chloroacetamidoDITMQ exhibited weak irreversibly acting affinity properties on this system.

Figure 31 shows the effect of incubating acetamidoDITMQ, chloroacetamidoDITMQ and isothiocyanatoITMQ over a range of 2-45 mins at various concentrations with hu-β₂-AR-CHO in these washout experiments. At concentrations upto 30Kᵢ, the irreversible binding of all the three compounds to the receptor appeared to improve with incubation periods of up to 15 mins, while slight recovery in ICYP binding occurred at an incubation period of 45 min. However, this trend was abolished when the compounds were incubated at higher concentrations (100-fold of their Kᵢ values) over this time range. Thus, there seems to be a more distinctive concentration dependence for the irreversible binding of isothiocyanato and chloroacetamido analogs as compared to the dependence of binding on period of incubation with the compounds. This was more the case with the isothiocyanato analog, where although slightly greater effect was observed upon incubation for 15 mins, shorter time periods of 2 and 5 mins were sufficient to demonstrate “irreversible” binding of the compound to the hu-β₂-AR. Collectively, the concentration and time data indicate that isothiocyanatoITMQ is an effective affinity analog on the hu-β₂-AR with very rapid reaction kinetics.
4.3.2. Protection by Competing β-AR Ligands and Nucleophiles against Affinity Binding

The ability of various β-AR ligands including (-)-isoproterenol (agonist), propranolol (antagonist) and acetamidoDITMQ (agonist, and close structural reversible binding analog) to protect against the irreversible binding of isothiocyanatoDITMQ to the hu-β_2-AR, was evaluated. This affinity analog was used at concentrations of 3- and 10-Kᵢ, and the β-AR protecting agents were used at molar concentrations of 30- to 300-fold their Kᵢ. As shown in figure 32 no changes in ICYP binding to the receptors was observed following treatment with the affinity analog in the presence of these protecting agents. In the absence of the affinity ligand, a nearly complete washout of the protecting agents was observed in these studies. These results indicate that preincubation with the reversibly acting β-AR ligands failed to prevent the irreversible binding of isothiocyanato analog to the receptors.

On the other hand, studies with competing amino acid nucleophiles differed from previous studies of chloroacetamidoDITMQ on the rat-β₃-AR. Both, glutathione and L-cysteine demonstrated concentration-dependent protection against the affinity binding of isothiocyanatoDITMQ to the hu-β₂-AR. Figure 33 shows the improvement in ICYP binding to the receptors in the presence of 0.01 and 0.1 M concentrations of these two nucleophiles. The protection was more significant at the 10 Kᵢ concentrations of the affinity compound, as compared to the 3 Kᵢ concentration. In control experiments, ICYP binding was not significantly reduced after washout of the nucleophiles, which implies that there may be a competition of binding with the affinity

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compound rather than at the receptor level. Figure 34 summarizes the results of protection assays with competing β-AR ligands and nucleophiles against irreversible binding of isothiocyanatoITMQ at 10-fold its $K_r$ concentrations. The degree of protection offered should be observed as a net increase in ICYP binding to the receptors upon treatment with the affinity compound in the presence of various protecting agents. As mentioned above, the β-AR agonists and antagonist (propranolol) failed to protect, whereas significant protection was offered by the nucleophiles, glutathione and L-cysteine.

4.3.3. Effect of Ligand Concentration and Exposure Time to UV Light on Photoaffinity Binding

When CHO cells expressing the hu-β₂-AR were incubated with various concentrations of azidoITMQ under red light for 1 hour and exposed to UV light for a fixed amount of time (0.5 and 5 mins), a concentration-dependent inhibition of ICYP binding to these cells was observed, indicating that the azido analog was photolyzable and bound irreversibly to this receptor (figure 35). At higher concentrations (10- and 100-$K_r$) of the photoaffinity ligand, inhibition of ICYP binding reached 47-87% of the total binding and was dependent upon the sample exposure times to UV light of 350 nm. The extent of photoaffinity ligand binding was directly proportional to its concentration and time period of exposure to UV light. Samples incubated with the azido analog under red light but not exposed to UV light did not exhibit irreversible binding, which implies that the exposure of the azido analog to UV light is necessary for the formation of the highly reactive nitrene species under these
photolytic conditions, which covalently binds to the receptor protein. In control experiments where CHO cells were exposed to UV light for 5 min in the absence of the photoaffinity ligand, the radioligand binding of ICYP was not significantly affected (figure 36). Similarly, insignificant differences in the extent of ICYP binding were observed between samples incubated in complete dark environment and those under red light for an hour.

4.3.4. Protection by Competing β-AR Ligands and Nucleophile against Photoaffinity Binding

When CHO cells expressing the hu-β2-AR were incubated with azidolTMQ (10K) in the presence of 100- to 300-Ki concentrations of the competing β-AR ligands, (-)-isoproterenol, propranolol or acetamidoDITMQ and irradiated with UV light, a significant increase in radioligand binding of ICYP to the cells was observed. These results of the protecting ligand effects, together with the corresponding controls, are shown in figure 37. In contrast to studies with chloroacetamidoDITMQ and isothiocyanatolTMQ, the β-AR ligands protected against photoaffinity binding of the azido analog to the receptors. On the other hand, following similar washout protocol, L-cysteine, at 0.1 mM, was only marginally able to increase the ICYP binding (figure 38). These results indicate that structural analogs occupy the same binding pocket as the photoaffinity ligand. This contrasts to the results observed with the reactive affinity analogs, and suggests that there are differences in the mechanisms of irreversible binding of the analogs possessing alkylating groups (haloacetamido and isothiocyanato) versus the photoaffinity (azido) analog.
4.4. Discussion

Among β-ARs, the most extensively characterized receptor subtype is the β₂-AR (Strader et al., 1995). Affinity and photoaffinity characterization of this receptor has typically involved the use of antagonist analogs (Lavin et al., 1982; Bergermeister et al., 1982; Dohlman et al., 1988; Molenaar et al., 1988; Ruoho et al., 1994). Few reports of irreversible interactions of agonists for β-adrenergic receptors have appeared. The TMQ analogs used in the present studies constitute a unique group of agonist compounds which are suggested to interact with the receptor at multiple sites, and because of the structural similarities to catecholamines, will overlap to some degree with binding sites common to those of classical agonists and antagonists. However, the failure of these classical β-AR ligands to protect against the irreversible binding of isothiocyanatoTMQ to the receptor also indicates that the affinity analog may be interacting with the receptor at an "exo-site" which is located outside that of the classical β-AR ligand-binding pocket, but close enough to influence the binding of ICYP to the receptor. Such a proposal of the exo-receptor hypothesis has been suggested for the interaction of salmeterol with the β-AR (Johnson et al., 1993).

The affinity behavior of isothiocyanatoTMQ on the hu-β₂-AR is similar to that observed with chloroacetamidoDITMQ on the rat-β₂-AR. On both receptor subtypes, this compound exhibited a concentration dependent irreversible binding, which lacked a significant time dependence, and was not protected in the presence of competing β-AR ligands. Thus, both affinity analogs appear to share a common mode of interaction with the receptors.
However, some differences between the chloroacetamido and isothiocyanato analogs were noted. Unlike its effect on the rat β3-AR (see chapter 2), the chloroacetamido analog exhibited weak affinity properties on the human β2-AR, whereas the isothiocyanato derivative inhibited ICYP binding to a significantly greater extent. Several explanations may be possible for these differences. The presence of two iodine atoms and chloroacetamido group on the chloroacetamidoDITMQ molecule versus the single iodine and smaller NCS moiety on isothiocyanatoITMQ may lead to considerable difference in bulk of the two compounds. Based on structural analyses of the β2-AR, it is believed that the binding pocket composed of tms 1, 2, 6 and 7 is rather constricted due to presence of several amino acids with extended side-chains in this region. Thus, it is possible that the less bulky isothiocyanatoITMQ fits better into the receptor binding pocket, whereas docking of the bulkier chloroacetamido analog in this region is hampered. The β3-AR, on the other hand, is hypothesized to possess amino acids with smaller side-groups forming a wider binding pocket in this region, which may accommodate the bulkier analog and provide a better fit, reflected in comparatively superior affinity properties of the bulky analogs relative to the β2-AR. Alternatively, it is possible that the greater effectiveness of inhibiting ICYP binding by the isothiocyanato analog relative to the chloroacetamido analog may be a reflection of the higher chemical reactivity of the isothiocyanato group as compared to the latter. This is indicated by the results of stability studies (see Appendix C). Protection with nucleophiles against irreversible binding of the isothiocyanato, but not the chloroacetamido analog, also corroborates these
differences in reactivities of the two analogs. It is known that isothiocyanates react rather rapidly with amino groups, whether or not they are attached to affinity groups (Langdon, 1977). The quenching of the reactivity of the electrophilic isothiocyanato compound by nucleophiles may be interpreted thereby as non-specific interaction. However, concentration-dependent protection of isothiocyanatoITMQ-induced inhibition of specific radioligand binding to the β₂-AR by the nucleophiles does provide indirect evidence for the covalent interaction of the affinity ligand with the β-AR.

In the photoaffinity experiments with azidoITMQ on human β₂-ARs, protection was observed in the presence of each of the competing β-AR ligands ((-)-isoproterenol, propranolol and acetamidoDITMQ). These results differ from the observed absence of protection by these ligands on the affinity binding of chloroacetamidoDITMQ and isothiocyanatoITMQ to β₁- and β₂-ARs, respectively. These differences may be related to the mechanisms of irreversible binding of the reactive affinity versus photoaffinity TMO analogs to these receptors. One advantage of using a photoaffinity ligand involves the lack of reactivity of the ligand until the time it is exposed to light of an appropriate wavelength and converted to a photoactive species which binds covalently to amino acids of the receptor located within the microenvironment (Guillory, 1989). Thus, in the absence of light, after co-incubation and equilibration of the azido analog and the protecting agent with the β₂-AR, the latter is able to successfully compete with the reversible binding of the azido analog at the receptor, assuming that the lifetime of the photoactive species is very short. The significant protection by the β-AR agonists and antagonist
strongly suggests that the photoaffinity analog binds to a site on the receptor common to these compounds. In this regard, isoproterenol, like other classical β-AR agonists, is believed to occupy the ligand binding region surrounded by tms 3,4 and 5. Antagonists like propranolol have been proposed to exist in multiple conformations, with the folded conformation within tms 1,6 and 7 being most favored (Hockerman et al., 1996). As discussed earlier, acetamidoDITMQ is hypothesized to occupy both the regions of the ligand binding pocket: the catechol portion interacting with amino acids in tms 3,4 and 5, whereas the 1-benzyl side-chain located within the region surrounded by tms 1,2,6 and 7. AzidoTMQ, a close analog of the acetamido derivative, would also be assumed to occupy both the sites within the β-AR ligand binding pocket and therefore face competition from isoproterenol, propranolol as well as acetamidoDITMQ for binding to the β-AR. Thus, photoaffinity labeling of the β-ARs with radiolabeled analog of azidoTMQ in the absence and presence of competing agonists or antagonists would provide useful clues about the sites of interaction of the compound with the receptor and enable characterization of the binding site of the 1-benzyl side-chain of TMQ analogs with the various β-AR subtypes.
Figure 30. Concentration-dependent inhibition of [$^{125}$I]ICYP binding to human $\beta_2$-AR in CHO cells in washout experiments after incubation with selected TMQ analogs for 2 min (□), 5 min (△), 15 min (○) or 45 min (○). Values are mean ± SEM of 3 experiments, each in triplicate. Panel A: AcetamidoDITMQ, Panel B: ChloroacetamidoDITMQ, Panel C: IsothiocyanatoITMQ.
Figure 30.
Figure 31. Effect of time of incubation with ligands at molar concentrations of 3K$_i$ (□), 10 K$_i$ (△), 30 K$_i$ (◇) and 100 K$_i$ (○) in washout experiments, upon the inhibition of [${}^{[125]}$I]ICYP binding to human β$_2$-AR in CHO cells. The data are mean ± SEM of 3 experiments, each in triplicate. Panel A: AcetamidoDITMQ. Panel B: ChloroacetamidoDITMQ. Panel C: IsothiocyanatolTMQ.
Figure 31.
Figure 32. Effect of selected β-AR ligands as protecting agents on the concentration-dependent inhibition of $[^{125}]$ICYP binding by isothiocyanatolTMQ to human β$_2$-AR in CHO cells. The data are mean ± SEM of 3 experiments, each in triplicate. Protecting agent in Panel A: (-)-Isoproterenol Panel B: Propranolol Panel C: AcetamidoDITMQ. Key. IsothiocyanatolTMQ (open bars), isothiocyanatolTMQ + protecting agent (diagonal bars; (-)-isoproterenol and propranolol at 30-fold excess and acetamidoDITMQ at 10-fold excess $K_i$ concentrations), and protecting agent only (speckled bar: acetamidoDITMQ at 30 $K_i$; checkered bars: (-)-isoproterenol and propranolol at 300-$K_i$, acetamidoDITMQ at 100 $K_i$).
Figure 33. Effect of nucleophilic amino acids on the concentration-dependent inhibition of [125I]ICYP binding by isothiocyanatoITMQ to human β2-AR in CHO cells. The data are mean ± SEM of 3 experiments, each in triplicate. Key. IsothiocyanatoITMQ (open bars); isothiocyanatoITMQ + 0.01 M nucleophile (forward diagonal bars); isothiocyanatoITMQ + 0.1 M nucleophile (reverse diagonal bars); 0.1 M nucleophile only (checkered bars). Panel A: Glutathione, Panel B: L-Cysteine.
Figure 34. Summary of protecting effect of competing $\beta$-AR ligands and nucleophiles against affinity binding of isothiocyanatoTMQ ($10K_\text{J}$) to human $\beta_2$-AR in CHO cells. Protection is indicated by a positive value of % change in total specific-$[^{125}]$ICYP-bound receptors. The data are mean ± SEM of 3 experiments, each in triplicate.
Figure 35. Concentration-dependent inhibition of $[^{125}\text{I}]$ICYP binding to human $\beta_2$-AR in CHO cells in washout experiments by azidolTMQ after incubation in red light for 1 hour followed by exposure to UV light at 350 nm for zero min ($\circ$), 30 secs ($\blacksquare$) or 5 mins ($\bullet$). Data are mean ± SEM of 2-6 experiments, each in triplicate.
Figure 36. Binding of $[^{125}\text{I}]$ICYP to human $\beta_2$-AR in CHO cells incubated for 1 hour in the dark (speckled bar) or in red light (checkered bar) followed by exposure to UV light of 350 nm for 5 mins. Also shown is binding of the radioligand to these cells incubated with azidoTMQ for 1 hour in dark (open bars) or in red light (diagonal bars) but not exposed to UV light. The data are mean ± SEM of 2-4 experiments, each in triplicate.
Figure 37. Effect of selected β-AR ligands as protecting agents on inhibition of [¹²⁵I]ICYP binding by azidolTMQ to human β₂-AR in CHO cells following incubation in red light for 1 hour. The data are mean ± SEM of 3-5 experiments, each in triplicate. Key. 10 Kᵢ AzidoTMQ, with photolysis for 30 secs (open bars); 10 Kᵢ azidoTMQ + protecting agent, with photolysis for 30 secs (diagonal bars); 10 Kᵢ azidoTMQ + protecting agent in absence of photolysis (speckled bars); and protecting agent alone, with photolysis for 30 secs (checkered bars). The protecting agents were: Panel A: (-)-Isoproterenol at 300 Kᵢ, Panel B: Propranolol at 300 Kᵢ, and Panel C: AcetamidoDITMQ at 100 Kᵢ.
Figure 37.
Figure 38. Effect of L-cysteine on inhibition of $[^{125}I]$ICYP binding by azidoITMQ to human β₂-AR in CHO cells following incubation for 1 hour in red light. The data are mean ± SEM of 3-4 experiments, each in triplicate. Key. 10 K, AzidoITMQ with photolysis for 30 secs (open bars); 10 K, azidoITMQ + 0.1 mM L-cysteine, with photolysis for 30 secs (diagonal bars); 10 K, azidoITMQ + 0.1 mM L-cysteine in absence of photolysis (speckled bars); and 0.1 mM L-cysteine alone, with photolysis for 30 secs (checkered bars).
CHAPTER 5

SUMMARY AND CONCLUSIONS

The long term goal of this project is to design potent, selective $\beta_3$-adrenoceptor ($\beta_3$-AR) selective analogs that belong to the tetrahydroisoquinoline class of compounds. The prototypical agent of this novel chemical class is trimetoquinol (TMQ, 1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxytetrahydroisoquinoline). TMQ differs from classical catecholamine $\beta$-AR agonists such as isoproterenol, in that it doesn’t contain a corresponding $\beta$-hydroxy group, has a cyclized semirigid tetrahydroisoquinoline ring, and possesses a large benzyl substituent at the 1-carbon position. The presence of this large 1-benzyl ring substituent is important for the $\beta$-AR activity of this compound (Lee et al., 1974, Feller et al., 1975). This drug possesses high potency equivalent to or greater than isoproterenol; however, like isoproterenol, it lacks selectivity among the three pharmacological subtypes of $\beta$-ARs. The development of highly selective $\beta_3$-AR agonists should be useful in the treatment of obesity, non-insulin-dependent diabetes, and gastrointestinal hypermotility disorders.
Studies in our laboratory have indicated the importance of the 1-benzyl side chain of TMQ in agonist activities on the β-AR subtypes. This led to the design of several 4'-substituted 1-benzyl analogs of TMQ, including both reversible and irreversible (affinity and photoaffinity) analogs which might serve as useful tools for characterization of the "hydrophobic" ligand binding pocket on the β-AR. It was established in earlier studies that the presence of one or two iodo groups on the 3'-and 5'-positions of the 1-benzyl ring of TMQ increased β3-AR selectivity (Konkar et al., 1996). Accordingly, the present study was undertaken with a series of 4'-substituted TMQ analogs possessing a 3'-monoiodo or 3',5'-diido groups on the 1-benzyl ring.

Pharmacological evaluations using biological tissues are compromised due to the presence of competing β-AR subtypes that may be present. Therefore, in order to overcome this potential limitation, homogeneous populations of human and rat β-AR subtypes stably expressed in Chinese hamster ovary (CHO) cells were utilized in most of the experiments. For the proposed studies, both receptor binding (affinity) and biochemical potency for the stimulation of adenylyl cyclase activity (cAMP accumulation) were determined for the TMQ analogs. Important information obtained from pharmacological and affinity studies of these compounds on the β-AR subtypes on these model cell systems and in isolated tissues is summarized as follows:

1. Many of the 1-benzyl ring 3',4'-di or 3',4',5'-tri-substituted analogs of TMQ possessed higher binding affinities and agonist potencies on the rat β3-AR and on the human β-AR subtypes as compared to (-)-isoproterenol. These
properties on the β₃-AR were comparable to those of BRL 37344. Moreover, while BRL 37344 was a partial agonist on the human β₃-AR, the TMQ analogs were full agonists.

2. An increase in lipophilicity and bulk at the 3',4',5'-positions of the 1-benzyl ring of TMQ improved binding affinities and agonist potencies on the rat β₃-AR whereas substitution of catechol hydroxyls with dimethoxy or methylenedioxy groups attenuated these properties. The 4'-acetamido-3',5'-diiodoTMQ analog possessed highest affinity and was among the most potent agonist analogs on the rat β₃-AR in CHO cells. The agonist potencies of the 4'-acetamido-3',5'-diiodoTMQ and other 3',5'-diiodo 1-benzyl ring substituted TMQ analogs were in the nanomolar and subnanomolar range on the rat and human β₃-ARs, and were 5- to 30-fold more potent than (−)-isoproterenol.

3. The potencies of 4-acetamido- and 4'-α-chloroacetamido-3',5'-diiodoTMQ for receptor-mediated activation of adenylyl cyclase in CHO cells expressing the β-AR were comparable to their functional potencies in isolated rat tissues. While these TMQ analogs were partial agonists for chronotropic response in right atria (β₁-AR) and for relaxation of trachea (β₂-AR), they were full agonists for relaxation of esophageal smooth muscle (atypical-β₁/β₂-AR) of rat. In addition, these analogs did not stimulate or antagonize α-ARs of rat aorta.

4. The 3'-iodo- and 3',5'-diiodo analogs of TMQ exhibited high binding affinities and potent agonist activities on each of the human β-AR subtypes. This was not unexpected due to presence of the catechol group (6,7-dihydroxy function) on the molecules. The binding affinities of these
compounds as well as (-)-isoproterenol were about 3- to 110-fold higher for the \( \beta_1 \)- and \( \beta_2 \)-ARs as compared to the \( \beta_3 \)-AR, while the agonist potencies were about 3- to 75-fold higher for the \( \beta_1 \)- and \( \beta_2 \)-ARs as compared to the \( \beta_3 \)-subtype. These results were in agreement with the reported lower affinities and agonist potencies of catecholamines and other classical agonists for the atypical-\( \beta \)/\( \beta_3 \)-AR as compared to the other two subtypes (Strosberg, 1995).

More significantly, the agonist potencies of the TMQ analogs on the \( \beta_3 \)-AR were in the nanomolar and subnanomolar range, comparable to other \( \beta_3 \)-AR selective agonists reported in literature (Strosberg, 1995). However, the potencies of these compounds on the \( \beta_1 \)- and \( \beta_2 \)-ARs were also in the picomolar range, indicating that they lack \( \beta \)-AR subtype selectivity.

5. Among the TMQ analogs, 4'-azido-3'-iodoTMQ was the most potent agonist on the three human \( \beta \)-AR subtypes. The potencies for activation of adenylyl cyclase on the three subtypes ranged from 13 - 48 pM, the highest being on the \( \beta_2 \)-AR subtype, despite its significantly lower binding affinity for this receptor as compared to the corresponding 3',5'-diiodo-substituted analogs.

6. The ratio of agonist potency : binding affinity (i.e. \( K_{act} : K_i \)) may provide information about the relative coupling efficiency of receptors following agonist activation. While these ratios on the three human \( \beta \)-AR subtypes ranged from 30 to 400 for the 3',5'-diiodo-substituted TMQ analogs, 4'-isothiocyanato-3'-iodoTMQ, and BRL 37344, higher coupling efficiencies seem to result as a result of receptor activation by (-)-isoproterenol and 4'-azido-3'-iodoTMQ. The affinity to agonist potency ratios of these two compounds
were comparable on the β₁-AR (ratios around 900) and β₂-AR (ratios of 3,000 to 4,000). While (-)-isoproterenol mediated coupling efficiency of the human β₂-AR was modest (ratio of 180) 4'-azido-3'-idoTMQ provided maximal efficiency with a ratio of 5,100 on this receptor subtype.

7. Washout studies of selected TMQ analogs on rat β₃-ARs in CHO cells indicated that 4'-α-chloroacetamido and 4'-α- bromoacetamido 3',5'-diiodoTMQ analogs bound irreversibly to the receptors, whereas 4'-acetamido-3',5'-diiodoTMQ did not. The irreversible binding of the former compounds, as indicated by inhibition of iodocyanopindolol (ICYP) following washout, was concentration-dependent. The 4'-α- chloroacetamido derivative was more effective in binding irreversibly to the receptors, as compared to the 4'-α-bromoacetamido analog. No significant change with incubation time of the ligands (in the range of 1-40 min with 4'-α-chloroacetamido-3',5'-diiodoTMQ, and 2-90 min with bromoacetamido-3'-5'-diiodoTMQ) was observed in these studies, and the compounds seem to bind irreversibly to the receptors within 1-2 min, indicating very rapid reaction kinetics.

8. Persistence of the chronotropic response for a significantly longer time period following washout of 4'-α-chloroacetamido-3',5'-diiodoTMQ pretreated right atria of rat as opposed to those pretreated with (-)-isoproterenol or 4'-acetamido-3',5'-diiodoTMQ provided functional evidence of the irreversible binding of the affinity analog to the β-AR.

9. Failure of β-AR agonists like (-)-isoproterenol, BRL 37344 or 4'-acetamido-3',5'-diiodoTMQ to protect against the irreversible binding of 4'-α-chloroacetamido-3',5'-diiodoTMQ to rat β₃-ARs in CHO cells however, indicate
that the affinity ligand may be binding to the receptor at an "exo-site" outside of the classical ligand binding region, in a manner similar to that proposed for the binding of the long-acting β-AR agonist, salmeterol (Johnson et al., 1993). Failure of several nucleophiles including glutathione, L-cysteine and L-lysine to quench the reactivity of 4'-α-chloroacetamido-3',5'-diiodoTMQ and attenuate its irreversible binding to the receptor additionally suggests that the affinity analog may be binding to a "hydrophobic" binding pocket of the receptor.

10. While 4'-α-chloroacetamido-3',5'-diiodoTMQ was an effective affinity ligand on the rat β3-AR, it was a relatively weaker inhibitor of ICYP in washout experiments on the human β2-AR. On the other hand, 4'-isothiocyanato-3'-iodoTMQ demonstrated significant irreversible binding to this β-AR subtype. The weaker affinity binding properties of 4'-α-chloroacetamido-3',5'-diiodoTMQ on the β2-AR subtype as compared to 4'-isothiocyanato-3'-iodoTMQ, and to those on rat β3-AR, are in agreement with the hypothesis that the ligand binding pocket formed by tms 1, 2, 6 and 7 of the β2-AR is narrower compared to the β3-AR subtype due to presence of amino acids with bulkier side-chains in these regions of the former receptor subtype (Blin et al., 1993). Thus, apart from other possible differences in ligand-receptor interactions, these spatial differences in the proposed ligand-binding pocket may be hampering the docking of the 3',5'-diiodoTMQ analog to a greater extent relative to the 3'-mono iodo TMQ analogs.

11. As observed with affinity binding of 4'-α-chloroacetamido-3',5'-diiodoTMQ to the rat β2-AR, the irreversible binding of 4'-isothiocyanato-3'
iodoTMQ to the human β₂-AR was concentration-dependent whereas similar levels of inhibition of ICYP binding were seen with incubation time of this compound over the range of 2-45 min, indicating rapid reaction kinetics.

12. While the nucleophilic amino acids, glutathione and L-cysteine protected against the irreversible binding of 4'-isothiocyanato-3'-iodoTMQ to the human β₂-AR, providing indirect support for covalent interactions of the affinity ligand with the receptor, reversible-binding β-AR ligands such as (-)-isoproterenol, propranolol and 4'-acetamido-3',5'-diiodoTMQ failed to offer protection. This once again indicates that these TMQ affinity ligands may be binding with high reactivity to the receptor at an exo-site which is outside of the conventional ligand binding pocket, but overlapping with that of ICYP.

13. In contrast to the chemical affinity analogs, 4'-azido-3'-iodoTMQ demonstrated irreversible binding to the human β₂-AR that was dependent both on concentration as well as photolysis time. More importantly, competing reversibly-binding β-AR ligands including (-)-isoproterenol, propanolol and 4'-acetamidoDITMQ significantly protected the irreversible binding of the photoaffinity analog to the receptor. These results indicate that the site of interaction of the photoaffinity compound may be overlapping with those of the reversible protecting β-AR ligands. Besides, the differences in kinetics of competition of the protecting agents with the photoaffinity ligand prior to and during photolysis as compared to those with the chemically reactive affinity ligands may account for the observed differences in ability of the β-AR ligands to protect against the irreversible binding of 4'-α-chloroacetamido, 4'-isothiocyanato and 4'-azido derivatives of TMQ.
14. Schild regression analysis of propranolol inhibition of human β2-AR mediated cAMP accumulation in CHO cells by (-)-isoproterenol, 4'-acetamido-3',5'-diiodoTMQ and 4'-α-chloroacetamido-3',5'-diiodoTMQ, yielded different pA₂ values for the antagonist. Propranolol was at least 100-fold more potent inhibitor of agonist effects of the TMQ analogs than of (-)-isoproterenol. This important finding provides strong support to the hypothesis that the TMQ analogs interact with the receptor at more sites as compared to isoproterenol, and that propranolol shares more common sites on the receptor with the TMQ analogs than with isoproterenol. As isoproterenol, like other classical β-AR agonists, is known to interact with the β-AR at sites occupied predominantly by tms 3, 4 and 5 (Blin et al., 1993), the additional sites of interaction of the TMQ analogs, common to those of propranolol, are proposed to be in the region surrounded by tms 1, 2, 6 and 7 (Hockerman et al., 1996).

In summary, these pharmacological, biochemical, and affinity studies with the selected derivatives of TMQ substituted at the 3',4',5'-positions, have confirmed the importance of interactions of the 1-benzyl ring of the compound with various β-AR subtypes. While the tetrahydroisoquinoline backbone of TMQ is known to interact with sites mainly on tms 3, 4 and 5 of the receptor which are common to other classical β-AR agonists (Fraundorfer, 1993), these studies indicate that the benzyl side-chain may occupy the ligand binding pocket that overlaps with that of antagonists such as propranolol and iodoacetylpindolol. We propose that this ligand binding pocket is in the region surrounded by tms 1, 2, 6 and 7 which includes proposed sites of interactions
of antagonists (Hockerman et al., 1996) as well as of β₃-AR selective agonists (Blin et al., 1993).

Receptor labeling and analysis of the β-ARs with these affinity and photoaffinity analogs of TMQ may enable testing of this hypothesis and should provide information regarding amino acids on the receptor binding domains that are critical for interactions of the benzyl side-chain of TMQ. The availability of this information will be valuable for rational modifications on the tetrahydroisoquinoline backbone and the 1-benzyl ring substituents of TMQ, and aid in designing potent β₃-AR-selective analogs for therapeutic applications.
APPENDIX A

CHEMICAL DESCRIPTION AND ABBREVIATIONS OF TRIMETOQUINOL ANALOGS USED IN THE STUDIES
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Abbreviated Name</th>
<th>Salt Form</th>
<th>Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(3',4',5'-Trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>Trimetoquinol (TMQ)</td>
<td>HCl</td>
<td>381.86</td>
</tr>
<tr>
<td>1-(4'-Methoxy-3',5'-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>DITMQ</td>
<td>HCl.1/2 EtOH</td>
<td>596.63</td>
</tr>
<tr>
<td>1-(4'-Amino-3',5'-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>AminoDITMQ</td>
<td>2HCl.H₂O</td>
<td>613.06</td>
</tr>
<tr>
<td>1-(4'-Acetamido-3',5'-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>AcetamidoDITMQ</td>
<td>HBr.1/2(CH₂CH₂)₂O</td>
<td>670.13</td>
</tr>
<tr>
<td>1-(4'-α-Chloroacetamido-3',5'-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>ChloroacetamidoDITMQ</td>
<td>HBr</td>
<td>679.52</td>
</tr>
<tr>
<td>1-(4'-α-Bromoacetamido-3',5'-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>BromoacetamidoDITMQ</td>
<td>HBr</td>
<td>723.97</td>
</tr>
<tr>
<td>1-(3',5'-Diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>DemethoxyDITMQ</td>
<td>HBr</td>
<td>469.15</td>
</tr>
</tbody>
</table>

Table 7. continued on next page...
Continued from previous page.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Abbreviated Name</th>
<th>Salt Form</th>
<th>Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- (3’-lodo-4’-isothiocyanatobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>IsothiocyanatotMQ</td>
<td>HBr</td>
<td>519.19</td>
</tr>
<tr>
<td>1- (3’-lodo-4’-azidobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>AzidotMQ</td>
<td>HCl.1/2 EtOH</td>
<td>495.75</td>
</tr>
<tr>
<td>1- (3’,4’,5’-trimethoxybenzyl)-6,7-methyleneoxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>6,7-MethyleneoxyTMQ</td>
<td>HCl</td>
<td>393.86</td>
</tr>
<tr>
<td>1- (3’,4’,5’-trimethoxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>6,7-DimethoxyTMQ</td>
<td>HCl</td>
<td>409.91</td>
</tr>
<tr>
<td>1- (4’-Acetamido-3’,5’-Diodobenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>6,7-DimethoxyacetamidoDITMQ</td>
<td>HCl.1/2(CH$_3$CH$_2$)$_2$O</td>
<td>665.74</td>
</tr>
<tr>
<td>1- (Naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>---</td>
<td>HBr</td>
<td>386.29</td>
</tr>
<tr>
<td>1- (Naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>---</td>
<td>HBr</td>
<td>386.29</td>
</tr>
</tbody>
</table>

Table 7. Chemical description and abbreviated names of trimetoquinol analogs used in these studies.
APPENDIX B

CHEMICAL DESCRIPTION AND STRUCTURES OF SELECTED COMPOUNDS USED IN THE STUDIES
<table>
<thead>
<tr>
<th>Compound</th>
<th>Salt Form</th>
<th>Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Isoproterenol</td>
<td>(+)-Bitartrate.2H$_2$O</td>
<td>397.35</td>
</tr>
<tr>
<td>BRL 37344 or (R<em>R</em>)-(±)-4-[2'-{2-Hydroxy-2-(3-chlorophenyl)ethylamino}</td>
<td>Sodium salt.1.5 H$_2$O</td>
<td>412.86</td>
</tr>
<tr>
<td>propyl]phenoxyacetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>HCl</td>
<td>295.81</td>
</tr>
<tr>
<td>Forskolin or 7β-Acetoxy-1α,8β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one</td>
<td>---</td>
<td>410.50</td>
</tr>
<tr>
<td>Glutathione (reduced form)</td>
<td>Free acid</td>
<td>307.30</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>HCl.H$_2$O</td>
<td>175.60</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>HCl</td>
<td>182.60</td>
</tr>
<tr>
<td>[$^{125}$I]-(−)-3-iodocyanopindolol or [$^{125}$I]ICYP</td>
<td>Methanolic soln., Specific Activity of 2000 Ci/mmol, 1 mCi/ml.</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Chemical description, salt or solution form and molecular weight of compounds other than TMQ analogs used in the studies.
Figure 39. Chemical structures of iodocyanopindolol and propranolol used in the studies.
APPENDIX C

GROSS STABILITY STUDIES OF AFFINITY LIGANDS.

BromoacetamidoDITMQ.

The bromoacetamide affinity analog was dissolved in deuterated methanol (MeOH-\(d_4\)), and its nuclear magnetic resonance (NMR) spectrum was determined. The solution was allowed to stand at room temperature overnight, and a second NMR spectrum was determined. The two NMR spectra were identical, indicating no reaction with MeOH-\(d_4\). Furthermore, additional NMR spectra taken after 3 and 6 days revealed no change. These studies demonstrate that the bromoacetamide affinity ligand is stable in methanol (alcohol) at room temperature. Similar studies at elevated temperature (boiling methanol, 65°C - 30 min) gave similar results - no reaction with methanol.

IsothiocyanatoDITMQ.

Similar NMR studies with the isothiocyanato (NCS) compound, however, revealed a significant change in the NMR spectrum after standing overnight in MeOH-\(d_4\) at room temperature. Comparisons of the initial and overnight NMR spectra revealed about 10% conversion to the methanol adduct.
REFERENCES


Cheng Y and Prusoff WH, Relationship between the inhibition constant (Kᵢ) and the concentration of an inhibitor that causes a 50% inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* 22: 3099-3108, 1973.


Fraser CM, Chung FZ, Wang CD and Venter JC, Site-directed mutagenesis of β-adrenergic receptors: substitution of aspartic acid-130 by asparagine produces a receptor with high affinity binding that is uncoupled from adenylate cyclase. *Proc Natl Acad Sci USA* **85**: 5478-82, 1988.


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Jockers R, Da Silva A, Strosberg AD, Bouvier M and Marullo S, New molecular and structural determinants involved in beta 2-adrenergic receptor


Konkar AA, Pharmacological evaluation of the β-adrenoceptor subtype interactions of trimetoquinol (TMQ) and related analogs. Ph.D. Dissertation, The Ohio State University, Columbus, OH, 1996.

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Lands AM, Luduena FP and Buzzo HJ, Differentiation of receptors responsive to isoproterenol. Life Sci 6: 2241-9, 1967b.


Marullo S, Emorine LJ, Strosberg AD and Delavier-Klutchko C, Selective binding of ligands to β1, β2 or chimeric β1/β2-adrenergic receptors involves multiple subsites. *EMBO J* 9: 1471-6, 1990.


Sokolovsky M, Affinity and photoaffinity labeling of receptors. In: *Brain Receptor Methodologies. Part A. General Methods and Concepts. Amines and*


