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Novel Modulation of Adenylyl Cyclase Type 2

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For my parents

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ABSTRACT

Conley, Jason M. Ph.D., Purdue University, December 2013. Novel Modulation of Adenylyl Cyclase Type 2. Major Professor: Val J. Watts.

Adenylyl cyclase isoforms are distinctly modulated by G protein subunits and are therefore hypothesized to be uniquely regulated by proteins that influence G protein signaling. Activator of G protein signaling 3 (AGS3) is a G protein modulator that binds Gαi subunits in the GDP-bound state, implicating AGS3 as an important regulator of Gi-coupled receptor signaling. We studied the ability of AGS3 to modulate recombinant adenylyl cyclase (AC) type 1 and 2 signaling in HEK293 cells following both acute and persistent activation of the D_{2L} dopamine receptor (D_{2L}DR). AGS3 expression modestly enhanced the potency of acute quinpirole-induced D_{2L}DR modulation of AC1 or AC2 activity. AGS3 also promoted desensitization of D_{2L}DR-mediated inhibition of AC1, whereas desensitization of D_{2L}DR-mediated AC2 activation was significantly attenuated. Additionally, AGS3 reduced D_{2L}DR-mediated heterologous sensitization of AC1 and AC2. Our results suggest that AGS3 alters G protein signaling in a complex fashion that is effector-specific and dependent on the duration of receptor activation. The present work also addressed the role of Gβγ subunits in the development of D_{2L}DR-mediated sensitization of AC2. The molecular signaling

components that contribute to the development of heterologous sensitization are largely unknown, but G protein subunits are strongly implicated in this adaptive process. We utilized Gβγ subunit sequestering proteins, small molecule and peptide Gβγ signaling inhibitors, and pharmacological Gβγ effector and kinase inhibitors to study the role of Gβγ subunit signaling pathways in the development of AC2 sensitization in HEK293 cells. Our results suggest that Gβγ subunit signaling is necessary for D_{2L}DR-mediated sensitization of AC2. The multitude and diversity of Gβγ signaling pathways that may underlie AC sensitization prompted us to develop a high-throughput cAMP assay platform to facilitate future unbiased approaches for the study of AC sensitization, such as siRNA library screening. As an intermediate step to the development of such assays, we addressed the lack of potent and selective small molecule modulators of AC. Identification of chemical probes for AC2 is particularly important because there are no published genetic deletion studies and few small molecule modulators. Therefore, we developed and executed an intact-cell small molecule screening approach and subsequent validation paradigm for the discovery of AC2 inhibitors. The NIH clinical collections I and II were screened for inhibitors of AC2 activity, using PMA-stimulated cAMP accumulation as a functional readout. Active compounds were subsequently confirmed and validated as direct AC2 inhibitors using orthogonal and counter screening assays. The screening effort identified SKF-83566 as a selective AC2 inhibitor with superior pharmacological properties for selective modulation of AC2 when compared to currently available AC

inhibitors. The utility of SKF-83566 as a small molecule probe to study the function of endogenous ACs was demonstrated in C2C12 mouse skeletal muscle cells.

CHAPTER 1. INTRODUCTION

1.1 The foundation of second messenger signaling: A historical perspective

Cyclic AMP (cAMP) is the prototypical second messenger signaling molecule and the impact of cAMP signaling research is highlighted by the fact that four Nobel prizes have been awarded for the study of cAMP signaling components (Sutherland in 1971, Krebs and Fischer in 1992, Gilman and Rodbell in 1994, and Lefkowitz and Kobilka in 2012). The identification of cAMP was first reported in the late 1950s by Earl Sutherland and colleagues while studying the relationship of the hormones epinephrine and glucagon to the activation of liver phosphorylase (Rall et al., 1957; Sutherland and Rall, 1958). Specifically, hormone treatment resulted in activation of liver phsophorylase and a heat-stable molecule was isolated from the particulate fraction of the tissue homogenate following fractionation (Sutherland and Rall, 1958). Remarkably, the heat-stable factor was able to activate liver phosphorylase in the supernatant fraction, while hormone treatment had no effect on the liver phosphorylase in the supernatant fraction (Rall et al., 1957; Sutherland and Rall, 1958; Sutherland and Rall, 1960). Ultimately, the heat-stable factor was found to be cAMP and the enzyme that synthesizes cAMP was termed the adenylyl cyclase (AC) enzyme (Rall and Sutherland, 1958; Sutherland et al., 1962). Cyclic AMP was

considered a second messenger because their results suggested that the hormone-mediated activation of liver phosphorylase occurs in two distinct steps (Rall et al., 1957). For example, the first messenger (hormone) was unable to activate liver phosphorylase on its own, but stimulated AC to produce cAMP (second messenger), which was capable of activating liver phosphorylase in the supernatant fraction (Sutherland and Rall, 1960). It was subsequently realized that a variety of hormones and catecholamines were able to stimulate cAMP responses in multiple tissue types (and elicit specific physiological responses), suggesting a common mechanism of signal relay (Robison et al., 1968; Sutherland and Rall, 1960). Nonetheless, until the early 1970s, it was unclear if adenylyl cyclases were responsible for both hormone-binding and cAMP synthesis or if intermediates between hormone and adenylyl cyclase existed (Robison et al., 1968). The requirement of GTP for hormonal activation of AC was the first explicit suggestion that intermediate signal transducers were involved in hormone-mediated AC activity (Rodbell et al., 1971a; Rodbell et al., 1971b). It was further observed that a stimulatory regulator (the guanine nucleotide-binding protein, Gs) was necessary for significant adenylyl cyclase activity under physiological conditions and that binding of GTP to the G protein promoted its association with the catalytic moieties (Pfeuffer, 1979; Ross and Gilman, 1977). It was also demonstrated that agonist binding of receptor increased the receptor size as measured by gel exclusion chromatography, but was not due to interaction of the AC catalytic moieties, as they eluted independently. (Limbird and Lefkowitz, 1978). Soon after, it was then

demonstrated that agonist-activated receptor coupled with G protein (Limbird et al., 1980), leading to the conclusion that the G protein is a communicator between the agonist-activated receptor and the AC enzyme (Figure 1.1). These studies intimately link receptor and G proteins to AC/cAMP-mediated physiological responses and form the backbone of the signaling pathway mechanism for how a hormone or catecholamine ligand is transduced into a physiological response.

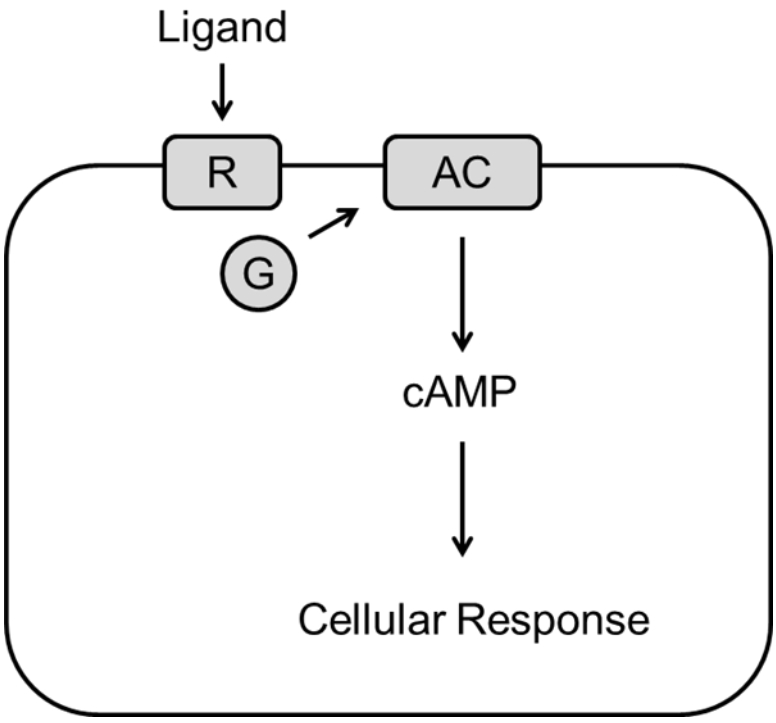

Figure 1.1 Schematic of hormone-stimulated cAMP signaling.

1.2 Adenylyl cyclase topology and catalytic mechanism

Early studies of cAMP suggested that the enzyme that catalyzes the formation of cAMP is membrane-bound (Sutherland and Rall, 1958). The topology of AC, however, was not realized until over three decades later, upon the cloning of the first AC isoform (Krupinski et al., 1989). Specifically, hydropathy analysis of the primary sequence suggested that the enzyme could be conceptually divided into two large sets of alternating hydrophobic and hydrophilic domains, where each of the hydrophobic domains consisted of six transmembrane spans and each of the hydrophilic domains was homologous to the cytoplasmic domain of guanylyl cyclase (Krupinski et al., 1989). The cloning of eight additional transmembrane AC isoforms suggested similar overall topology. The proposed structure of AC is thought to have a short, variable cytoplasmic N-terminus that is followed sequentially by the first six-transmembrane cassette (M1), the first large cytoplasmic domain (C1a and C1b), the second six-transmembrane cassette (M2), and finally another large cytoplasmic domain (C2a and C2b) (Figure 1.2) (Sunahara et al., 1996). The most conserved stretches of primary sequences reside in the N-terminal segments the large cytoplasmic domains, specifically in C1a and C2a, whereas the sequences are most divergent in the N-terminus and C1b and C2b domains (Sadana and Dessauer, 2009).

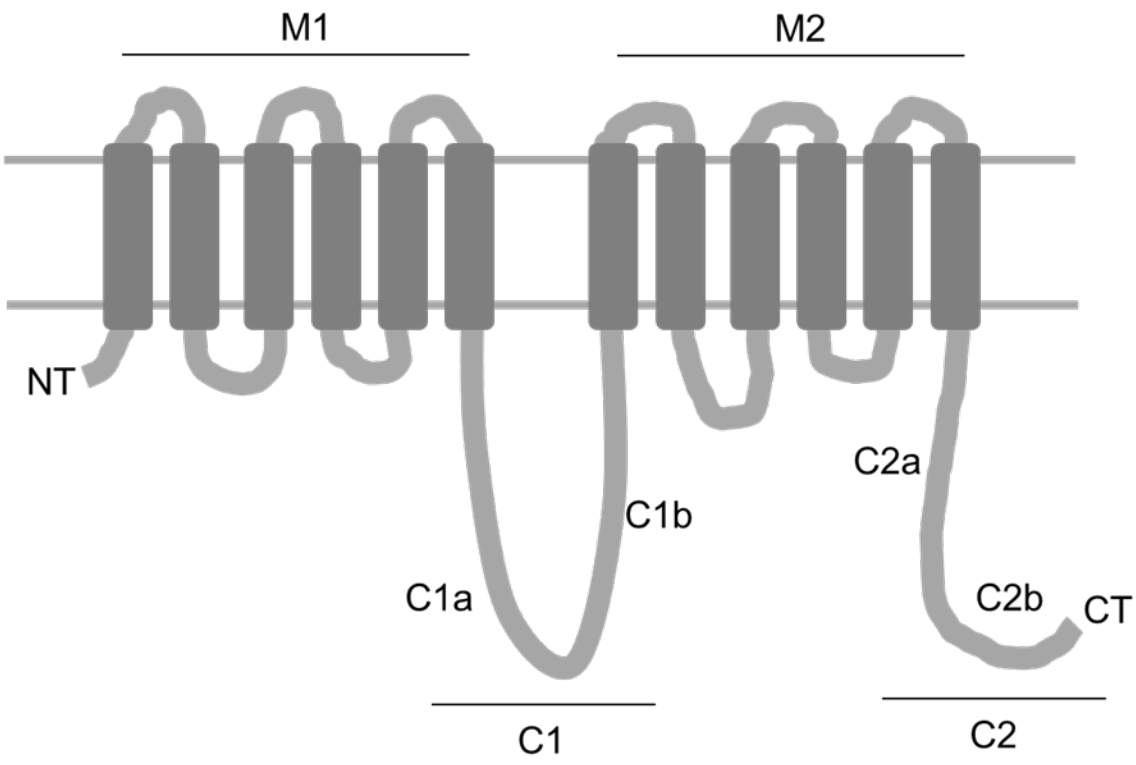

Figure 1.2 Topology of adenylyl cyclase.

A complete understanding of the structure of AC has remained elusive, as a crystal structure of an entire AC enzyme remains to be reported. However, much of what we know about the structure and function of AC stems from biochemical studies of the C1a and C2a cytoplasmic domains. For example, these regions can be expressed independently of other domains and possess catalytic activity (Dessauer and Gilman, 1996; Whisnant et al., 1996; Yan et al., 1996) and such biochemical studies of the catalytic domains provided the basis for the crystal structures of AC. Specifically, high resolution crystal structures of a C2 domain homodimer from AC2 (Zhang et al., 1997) and an AC5-C1a/AC2-C2a heterodimer (Tesmer et al., 1997) have been described. The crystal structures of the two cytoplasmic domains suggest that they interact to form a "pseudosymmetrical" structure that makes up the catalytic core of AC (Tesmer et al., 1997; Zhang et al., 1997). The interface of the C1a and C2a domains is expected to have conformational flexibility that allows for the relative orientation of the domains to change, while remaining associated (Tesmer et al., 1997). For example, the catalytic core is thought to exist in at least two states: an open state that is thought to be unable to accommodate ATP, and a closed state where domains collapse around ATP to form the active site (Tesmer et al., 1997). The mechanism of catalysis of ATP to cAMP is thought to be a two-metal-ion catalysis mechanism (similar to that utilized by Pol I DNA polymerases) where two metal ions coordinate with ATP in the active site to stabilize a pentavalent transition state, facilitating the conversion of ATP to cAMP (Tesmer et al., 1999). Additionally, the crystal structures of the catalytic domains of AC suggest that

Gαs and the small molecule AC activator, forskolin, (see below for discussion of AC modulation by forskolin) act allosterically to influence the orientation of the C1a/C2a interface, thus priming the active site for catalysis (Tesmer et al., 1999). In contrast, the Gαi subunit of the inhibitory G protein (Gi) is thought to allosterically influence the relative orientation of the C1a/C2a interface in an opposite manner, thereby inhibiting the collapse of catalytic residues around ATP (Dessauer et al., 1998; Tesmer and Sprang, 1998). The crystal structures of the AC catalytic domains, together with biochemical studies have provided a great deal of insight into the mechanism of AC modulation by forskolin, Gαs, and Gαi subunits. However, the nine membrane-bound ACs are distinctly modulated by many signaling molecules (including Gαi/o subunits, Gβγ protein subunits, Ca^{2+}, and protein kinases) that are downstream components of G protein coupled-receptor signaling (Hanoune and Defer, 2001). Each of these signaling molecules may affect catalysis by allosterically modulating the C1a/C2a interface, but the precise molecular details that contribute to the differential modulation of AC isoforms are currently not well understood (Tesmer and Sprang, 1998).

1.3 Overview of cAMP signaling: Adenylyl cyclase as a signal integrator within the cascades that link extracellular stimuli and the resulting cellular responses

The early studies of hormone-stimulated AC signaling identified the general G protein-mediated signal transduction mechanism by which

extracellular ligands can modulate cellular responses via cAMP second messenger signaling (i.e., receptor-binding of ligand stimulates G protein activation, resulting in AC activation and cAMP formation). These studies defined ACs as effectors of G protein-coupled receptor signaling. The decades following these seminal studies have provided a great deal of research that has built upon the backbone of G protein-mediated signal transduction to reveal a high level of complexity and specificity. ACs serve as general signal integrators, receiving input that is transduced from a variety of ligands that modulate diverse types of G protein-coupled receptors, thus providing cAMP as an intermediate signal between extracellular stimuli and cellular response.

The cellular levels of cAMP are directly modulated by two families of enzymes: adenylyl cyclases, that synthesize cAMP from ATP as described above (Hanoune and Defer, 2001), and phosphodiesterases (PDEs) that degrade cAMP (Bender and Beavo, 2006). PDE activity was described soon after the discovery of cAMP and AC activity (Butcher and Sutherland, 1962) and it is currently known that PDEs are phosphohydrolase enzymes that regulate the cellular levels of cyclic second messengers by catalyzing the hydrolysis of the 3’ cyclic phosphate bonds of cyclic adenosine- and guanosine-3’,5’-monophosphate (cAMP and cGMP) (Bender and Beavo, 2006). PDEs have been classified into 11 different families based on amino acid sequence homology and can be further grouped into three categories based on their substrate specificity (i.e., cAMP-specific, cGMP-specific, or dual specificity for cAMP and cGMP). There are 21 different gene products that have alternative transcriptional start sites and alternative

splicing of mRNA, yielding the possibility of >100 PDE protein products (Bender and Beavo, 2006). The many forms of PDE dynamically modulate the balance of cellular cAMP and have been linked to specific physiological processes (Bender and Beavo, 2006).

The ACs functionally integrate signals that are transduced from a variety of diverse extracellular stimuli into a single second messenger molecule, cAMP. Downstream of cAMP, the canonical signaling pathway proceeds by direct activation of cAMP-dependent kinase, also known as protein kinase A (PKA). As cAMP is the prototypical second messenger, PKA is the prototypical protein kinase and was identified in 1968 (Walsh et al., 1968). The PKA holoenzyme is formed by two regulatory subunits and two catalytic subunits from a selection of four regulatory subunit isoforms (RIα, RIβ, RIIα, and RIIβ) that are functionally non-redundant and three catalytic subunit isoforms (Cα, Cβ, and Cγ) (Taylor et al., 2012). Upon binding of two cAMP molecules to each regulatory subunit within a holoenzyme, the catalytic subunits dissociate and phosphorylate specific targets on serine and threonine residues that are found within a (RRX-S/T-X) PKA consensus motif (Kemp et al., 1977; Taylor et al., 2012; Ubersax and Ferrell, 2007). PKA signaling specificity is achieved by the complement of regulatory and catalytic subunit isoforms and PKA substrates that are expressed within a particular cell (Taylor et al., 2012). The signaling is further fine-tuned by the directed subcellular localization of PKA by direct binding to A-kinase anchoring proteins (AKAPs) (Welch et al., 2010). The AKAPs facilitate the formation of

macromolecular complexes that form PKA signaling hubs within specific cellular locations that contribute to diverse biological processes (Taylor et al., 2012).

PKA was initially thought to be the sole downstream effector of cAMP and remains a prevalent effector signaling system, but it has also become apparent that cAMP modulates additional downstream targets. For example, cyclic nucleotides directly modulate channels that conduct cations into the cytoplasm. Two structurally-related families of cyclic nucleotide modulated cation channels have been identified that differ in their mode of activation (Craven and Zagotta, 2006). Specifically, the cyclic nucleotide gated (CNG) cation channels are opened upon direct binding of cAMP or cGMP (Kaupp and Seifert, 2002). The CNG channels have been detected in several tissue types and are highly expressed in retinal photoreceptors and olfactory neurons, where they are known to be key mediators of visual and olfactory signal transduction (Kaupp and Seifert, 2002). The other family of cyclic nucleotide-modulated cation channels are the hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels and are mainly modulated by voltage (the channels open to cations upon hyperpolarization, but close due to depolarization), but the probability of channel opening is increased upon cyclic nucleotide binding (Craven and Zagotta, 2006). HCN channels are expressed in brain and heart tissue and are thought to underlie the cation current following hyperpolarization in excitable cells such as neurons and cardiac pacemaker cells (Biel, 2009).

In 1998, it was discovered that cAMP activates a family of guanine nucleotide exchange factors that were named exchange proteins activated by

cAMP (Epacs) (de Rooij et al., 1998; Kawasaki et al., 1998). Two Epac isoforms have been characterized, Epac1 and Epac2, and it is thought that Epacs catalyze the exchange of GDP for GTP on the ras-like GTPases Rap1 and Rap2 upon binding cAMP (Gloerich and Bos, 2010). Interestingly, Rap-independent Epac signaling has more recently been observed through a variety of additional signaling molecules including ras, phospholipase C, protein kinase D, and ERK1/2 (Billington and Hall, 2012). The expanding signaling functions of Epac proteins have gained attention for their contribution to cAMP signaling in the heart, brain, pancreas, vasculature, and lungs (Schmidt et al., 2013).

In summary, ACs are central mediators of the signal relay that is initiated by extracellular stimuli and results in a cellular response (Figure 1.3). Signaling input from a large number of G protein-coupled receptors is integrated by ACs and is further relayed in the form of cAMP. Cyclic AMP proceeds to modulate downstream effector proteins that include PKA, Epacs, and CNG/HCN channels that influence specific cellular responses that ultimately modulate diverse physiological processes including metabolism (Robison et al., 1968), cell growth and differentiation (Boynton and Whitfield, 1983; Friedman, 1976), learning and memory (Kandel, 2001), cardiac contractility (Drummond and Severson, 1979; Sobel and Mayer, 1973), and immune responses (Mosenden and Tasken, 2011; Peters-Golden, 2009; Serezani et al., 2008). The complete molecular details that contribute to the signaling specificity that allows for such diverse responses remain unclear, but are thought to be dependent on the complement of signaling

proteins that are expressed within a given cell and the molecular organization of these associated signaling components.

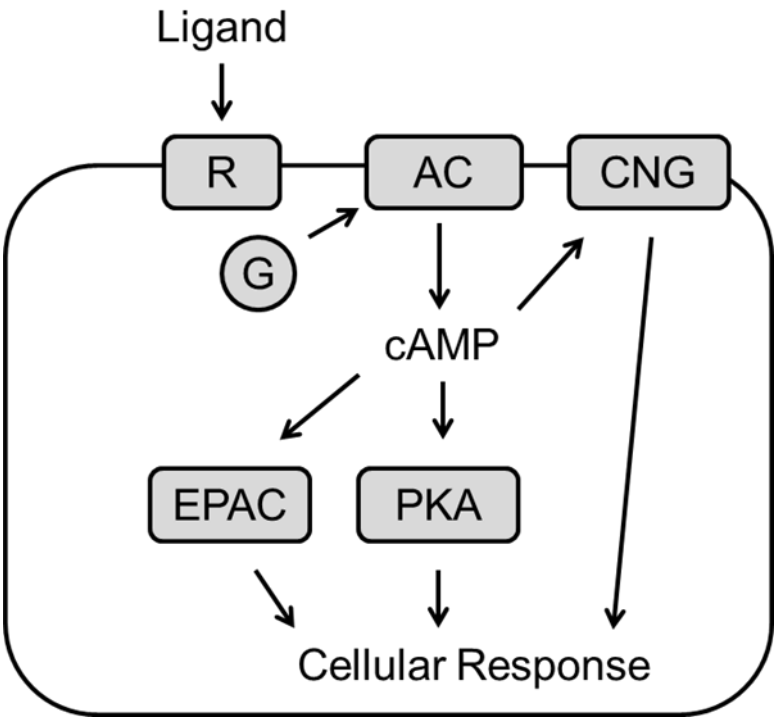

Figure 1.3 Position of adenylyl cyclase within the cAMP signaling pathway.

1.4 Adenylyl cyclase signaling properties: Contributions to cAMP signaling specificity

The specificity of cAMP signaling is tightly regulated, in part, by the repertoire and interplay of signaling molecules present within a given cell. Though ACs are only one part of the cAMP signaling pathway, a major contributing factor to this specificity is that nine membrane-bound mammalian AC isoforms have been identified, each with distinct patterns of regulation by G protein subunits, protein kinases, and Ca^{2+} (Hanoune and Defer, 2001; Patel et al., 2001). The modulation of cAMP signaling at the level of AC is considered in detail below.

1.4.1 G protein-coupled receptor signaling

1.4.1.1 G protein activation cycle

The G protein-coupled receptor family is a large (~1000 genes in the human genome) (Fredriksson et al., 2003) and versatile receptor family whose members contribute to nearly all physiological processes (Lefkowitz, 2004). G protein-coupled receptors bind extracellular ligands that induce conformational changes in the receptor that facilitate the activation of associated G protein heterotrimers by exchange of GDP for GTP on the G protein α subunit (Gilman, 1987). Upon activation, the G protein heterotrimer undergoes a conformational change that allows Gα and Gβγ subunits to dissociate/rearrange into an orientation that permits these subunits to modulate effector proteins (Coleman et

al., 1994; Lambright et al., 1994; Taussig et al., 1994). The G protein signaling cycle terminates upon hydrolysis of GTP to GDP on the Gα subunit, resulting in the inactivating reassociation/rearrangement of Gα and Gβγ (Figure 1.4) (Coleman et al., 1994; Mixon et al., 1995).

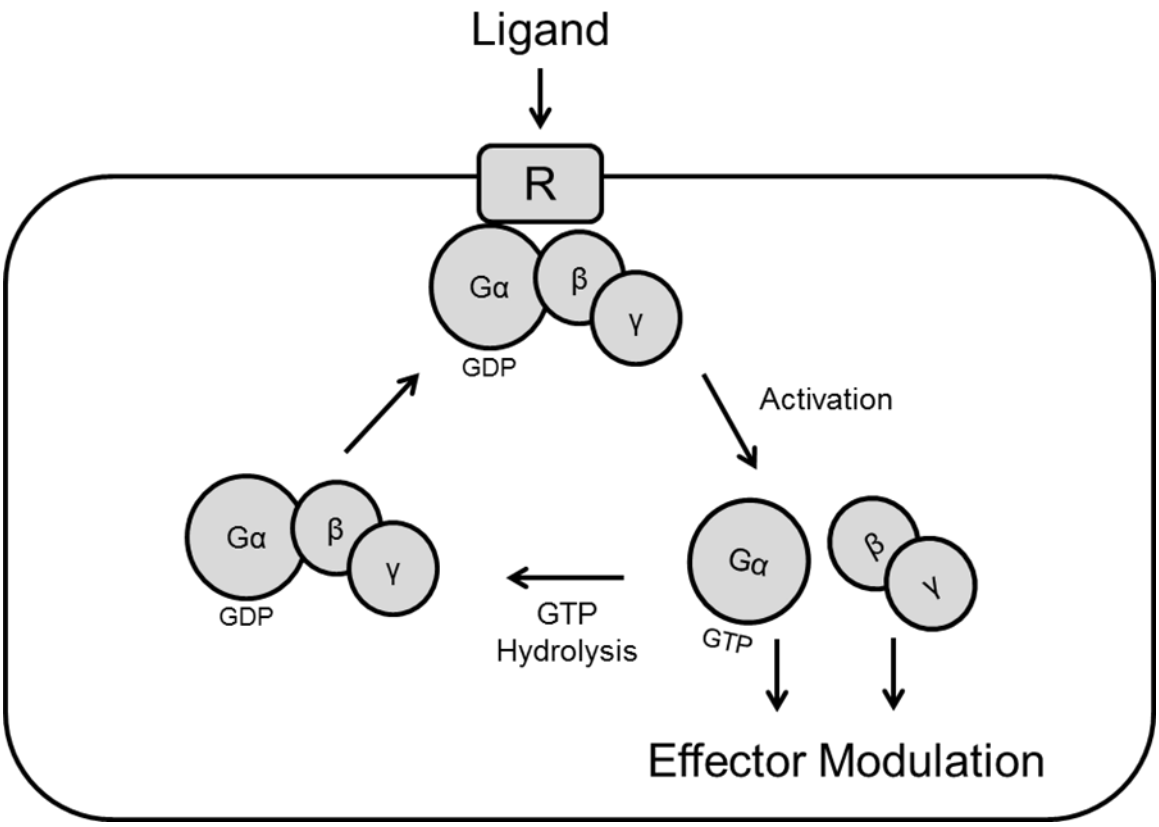

Figure 1.4 G protein activation cycle.

1.4.1.2 Effector modulation

Four families of Gα, five Gβ subunit isoforms, and twelve Gγ subunit isoforms have been identified (Hurowitz et al., 2000; Khan et al., 2013). Early studies implicated Gα subunits in effector modulation and it is known that the specific type of Gα subunit can contribute to the direct or indirect modulation of AC (Figure 1.5). For example, members of the Gαs family (the *GNAS* gene is thought to give rise to several gene products) (Bastepe, 2007) are thought to directly bind to AC in the C2a region (Tesmer et al., 1997; Yan et al., 1997) and stimulate AC activity (Iyengar, 1993). The inhibitory Gα subunits are comprised of $G\alpha i_{1, 2, \text{and } 3}$, Gαo, and Gαz and directly inhibit AC activity by interacting with the C1a domain (Dessauer et al., 1998), presumably preventing the “closed state” of the AC catalytic core (Tesmer and Sprang, 1998).

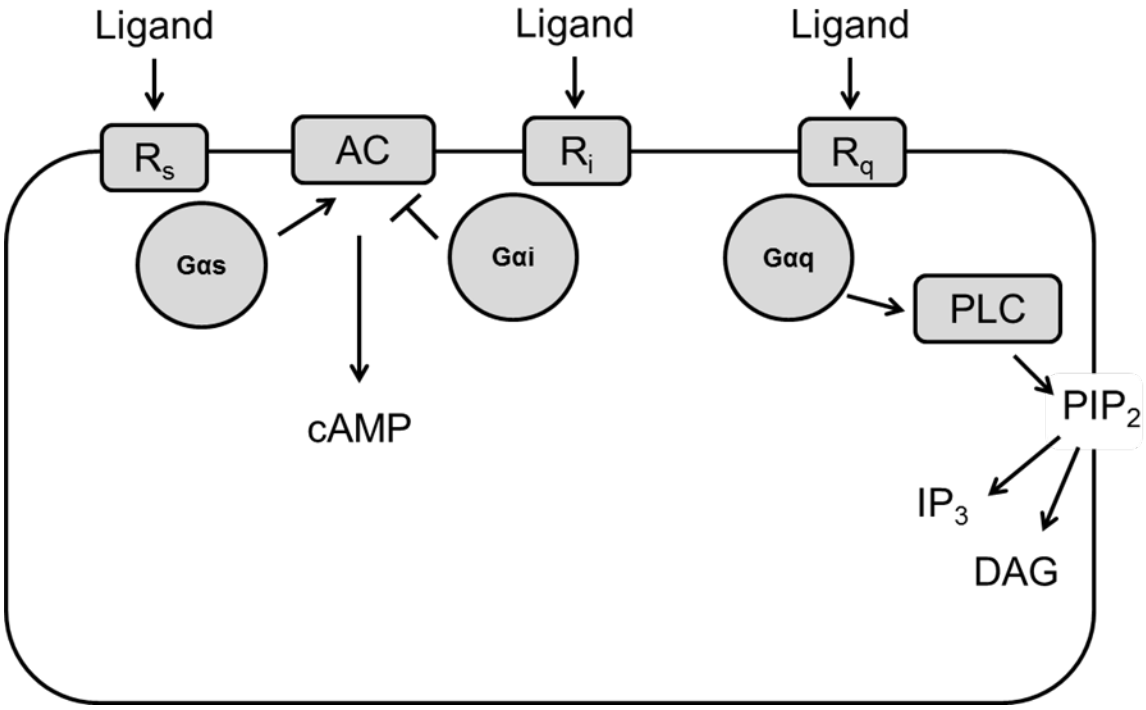

Figure 1.5 Gα subunit signaling.

The identification of Gα12 and Gα13 was first reported in 1991 (Strathmann and Simon, 1991) and Gα12/13 modulation of Rho guanine nucleotide exchange factor proteins (RhoGEFs) has been widely observed (Siehler, 2009). However, Gα12/13 has also recently been implicated in the direct and selective modulation of AC7 (Jiang et al., 2008; Jiang et al., 2013b). In contrast to Gαs, Gαi, and Gα12/13 (that directly modulate AC activity), Gαq/11 is thought to indirectly modulate AC activity. For example, Gαq/11 activates phospholipase C, which catalyzes the conversion of PIP_2 into IP_3 (binds to IP_3 receptors on the ER, causing Ca^{2+} release) and diacylglycerol (stimulates protein kinase C) (Hepler et al., 1993; Smrcka et al., 1991; Sternweis and Smrcka, 1992). Ca^{2+} and protein kinase C (PKC) uniquely modulate ACs in isoform-dependent manners (see regulatory properties).

Gβγ subunits were originally thought to be necessary for the inactivation of Gα subunits (Northup et al., 1983) and reassociation/rearrangement of the G protein heterotrimer, thus allowing receptor coupling and subsequent activation. However, the Gβγ subunits have since gained appreciation for direct modulation of a large number of effectors including ACs, phospholipase Cβ, Kir3 potassium channels, and voltage-gated Ca^{2+} channels and are being increasingly recognized for their contribution to diverse signaling pathways that are related to the different effectors that they modulate (for reviews, see (Khan et al., 2013; Smrcka, 2008)). Furthermore, genetic knockdown of individual Gβ and Gγ subunits in animal models suggest that specific Gβγ subunit isoforms contribute

to diverse biological functions including nociception, angiogenesis during embryonic development, and seizure susceptibility (Khan et al., 2013).

1.4.1.3 Modulation of G protein cycle by accessory proteins

In addition to G protein-coupled receptor modulation, the G protein activation cycle is sensitive to accessory proteins that include regulators of G protein signaling (RGS) and activator of G protein signaling (AGS) proteins (Blumer et al., 2007; Neubig and Siderovski, 2002). There are 20 distinct genes that have been identified for RGS family members (some with splice variants) that are categorized into four subfamilies (R4/B, RZ/A, R7/C, and R12/D) (Abramow-Newerly et al., 2006). All members of the RGS protein family have a conserved ~120 residue region that is known as the RGS domain and this region binds directly to GTP-bound Gα subunits to accelerate the rate of GTP hydrolysis, thereby terminating the G protein cycle (Abramow-Newerly et al., 2006; Neubig and Siderovski, 2002). The AGS proteins are a diverse family of 10 proteins (AGS1-10) that were identified in a functional yeast-based screen for receptor-independent signaling (Blumer et al., 2007). AGS proteins are divided into three groups based on their activity in the yeast-based screen and biochemical studies (Blumer et al., 2007). The Group I AGS protein (AGS1) displays guanine-nucleotide exchange activity similar to that of a G protein-coupled receptor. The remaining AGS proteins function independent of guanine-nucleotide exchange and are subdivided based on their interaction partners. Specifically, Group II AGS proteins (AGS3-AGS6) interact with Gα subunits and Group III AGS

proteins (AGS2, 7, 8, and 10) interact with Gβγ subunits. The RGS and AGS proteins influence G protein signaling and effector activation in diverse ways, including modulation of the rate of GTP hydrolysis and the availability and/or stability of Gα or Gβγ subunits (Blumer et al., 2007; Neubig and Siderovski, 2002; Sato et al., 2006).

1.4.2 Regulatory properties of adenylyl cyclase isoforms

The common regulatory property of all AC isoforms is that they are activated by the stimulatory G protein α subunit (Gαs), but otherwise display unique regulatory profiles that are modulated by multiple arms of the G protein-coupled receptor pathways. The AC isoforms are categorized into four subgroups based on their sequence similarities and specific regulatory properties (Hanoune and Defer, 2001; Patel et al., 2001).

1.4.2.1 Group I ACs

Group I ACs (AC1, AC3, and AC8) are collectively considered the Ca^{2+}/calmodulin-stimulated ACs. Ca^{2+}-bound calmodulin stimulates AC1 and AC8 via direct interactions (Cali et al., 1996; Choi et al., 1992a) within the C1b and C2 domains of AC1 and the N-terminus and C2 domains of AC8 (Gu et al., 2000; Simpson et al., 2006). In contrast to AC1 and AC8, AC3 appears to be conditionally activated by calmodulin, as it is dependent on activated Gαs or forskolin treatment (Choi et al., 1992b). Extensive studies of AC1 and AC8

suggest that these ACs are physiologically modulated by Ca^{2+} via a phenomenon known as capacitative calcium entry (CCE) (Willoughby and Cooper, 2007). CCE was first proposed in 1986 and is characterized by Ca^{2+} entering the cell through store-operated calcium channels in the plasma membrane in response to depletion of intracellular Ca^{2+} that is stored in the endoplasmic reticulum (Putney, 1986). It is thought that the proximity of ACs to the store-operated calcium channels contributes to the modulation of these ACs by local pools of Ca^{2+} (Willoughby and Cooper, 2007). Interestingly, AC1 appears to be more sensitive to Ca^{2+} stimulation than AC8, as Ca^{2+} more potently stimulates AC1 (Masada et al., 2009). Furthermore , the modulation of AC1 and AC8 by Ca^{2+} appears to be influenced by coincident signaling of Gαs because the convergent stimulation of AC1 by Gαs and Ca^{2+} influx is synergestic, but merely additive for AC8 (Cumbay and Watts, 2001; Nielsen et al., 1996). Though AC1 is relatively more sensitive to Ca^{2+} and Gαs signaling than AC8, studies suggest that AC1 is also more sensitive than AC8 to inhibition by Gαi in response to Gi-coupled receptor activation (Cumbay and Watts, 2001; Nielsen et al., 1996; Taussig et al., 1993a). Direct inhibition by Gβγ subunits is a common property of each of the group I ACs (Diel et al., 2008; Diel et al., 2006; Steiner et al., 2006; Tang and Gilman, 1991). Specifically, Gβγ subunits modulate AC1 signaling with a pattern that includes attenuation of AC stimulation in response to Gαs-mediated activation, whereas the inhibition observed by Gαi is enhanced (Tang and Gilman, 1991; Taussig et al., 1994).

The group I ACs are also notably regulated by protein kinases. For example, though Ca^{2+}-bound calmodulin is known to stimulate group I ACs, AC1 and AC3 are inhibited directly by calmodulin-dependent kinase (CaMK) phosphorylation. Specifically, AC1 is inhibited by CaMKIV (Wayman et al., 1996) and AC3 is inhibited by CaMKII (Wei et al., 1996), offering distinct modes of feedback inhibition through calmodulin, whereas AC8 does not appear to be sensitive to either CaMKII or CaMKIV phosphorylation (Wayman et al., 1996). It is also suggested that AC8 is insensitive to PKC modulation, whereas AC1 and AC3 are stimulated by phorbol ester treatment (Jacobowitz et al., 1993).

1.4.2.2 Group II ACs

Group II ACs (AC2, AC4, and AC7) are collectively insensitive to Ca^{2+} (submicromolar concentrations) and widely believed to be insensitive to Gαi/o modulation (Tang and Gilman, 1991; Taussig et al., 1993b; Taussig et al., 1994). As with all AC isoforms, each of the group II ACs are activated by Gαs stimulation. However, the group II ACs display unique profiles of modulation in response to PKC activation. AC2 is stimulated by PKC activation and has been the most extensively studied group II AC isoform with regard to PKC activation. For example, PKC-mediated stimulation of AC2 has been demonstrated both *in vitro* and in intact cell systems and has been observed in response to phorbol ester treatment (Jacobowitz and Iyengar, 1994; Shen et al., 2012; Yoshimura and Cooper, 1993; Zimmermann and Taussig, 1996) and via Gq-mediated PKC

activation (in response to Gq-coupled receptor activation and by constitutively active Gq (Q209L)) (Cumbay and Watts, 2005). A comprehensive study of every PKC isoform has not been reported, but PKCα and PKCδ have both been implicated in the activation of AC2 (Nguyen and Watts, 2006; Zimmermann and Taussig, 1996). Further solidifying the direct modulation of AC2 by PKC phosphorylation, a recent study mapped the phorbol ester- and muscarinic receptor-mediated PKC phosphorylation in HEK293 cells to S490 and S543 in the C1b domain (Shen et al., 2012). Similar to AC2, AC7 is stimulated by PKC activation in response to phorbol ester treatment (Hellevuo et al., 1995; Watson et al., 1994). Phorbol ester treatment also potentiates Gs-coupled receptor-mediated AC7 activity (Haslauer et al., 1998) and PKCδ is thought to mediate this potentiation response (Nelson et al., 2003). In contrast to AC2 and AC7, AC4 does not appear to be activated by PKC as tested in response to phorbol ester treatment and reconstituted PKCα (Jacobowitz et al., 1993; Zimmermann and Taussig, 1996). Further, Gαs stimulation and Gβγ-mediated potentiation of AC4 activity can be inhibited by PKCα activation (Zimmermann and Taussig, 1996).

In addition to PKC modulation of group II ACs, Gβγ subunits are thought to modulate the AC activity of all members of group II (AC2, AC4, and AC7) (Gao and Gilman, 1991; Tang and Gilman, 1991; Yoshimura et al., 1996). Interestingly, the Gβγ-mediated stimulation of group II ACs is thought to be conditional, as it is dependent on additional modes of AC stimulation (including Gαs- and PKC-mediated activation) (Tang and Gilman, 1991; Taussig et al.,

1993b; Taussig et al., 1994). These modulatory properties of group II ACs are well-established and have been demonstrated *in vitro* and in intact cell assays in response to Gi/o-coupled receptor activation. (Federman et al., 1992; Tang and Gilman, 1991; Tsu and Wong, 1996; Zimmermann and Taussig, 1996). Studies with AC2 are consistent with a direct modulation of AC2 activity, as multiple Gβγ interaction sites have been characterized within the C1a, C1b, and C2 domains of AC2 (See Figure 1.6) (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006; Weitmann et al., 2001). Though Gβγ subunits in response to Gi-coupled receptor activation provide enhancement of AC2 activity, recent evidence suggests that Gβγ subunits from Gq-coupled receptor activation provide modest inhibition of AC2 activity (Shen et al., 2012), suggesting bi-directional modulation of AC2 by Gβγ subunits that is dependent on the Gα subunit that defines the heterotrimer.

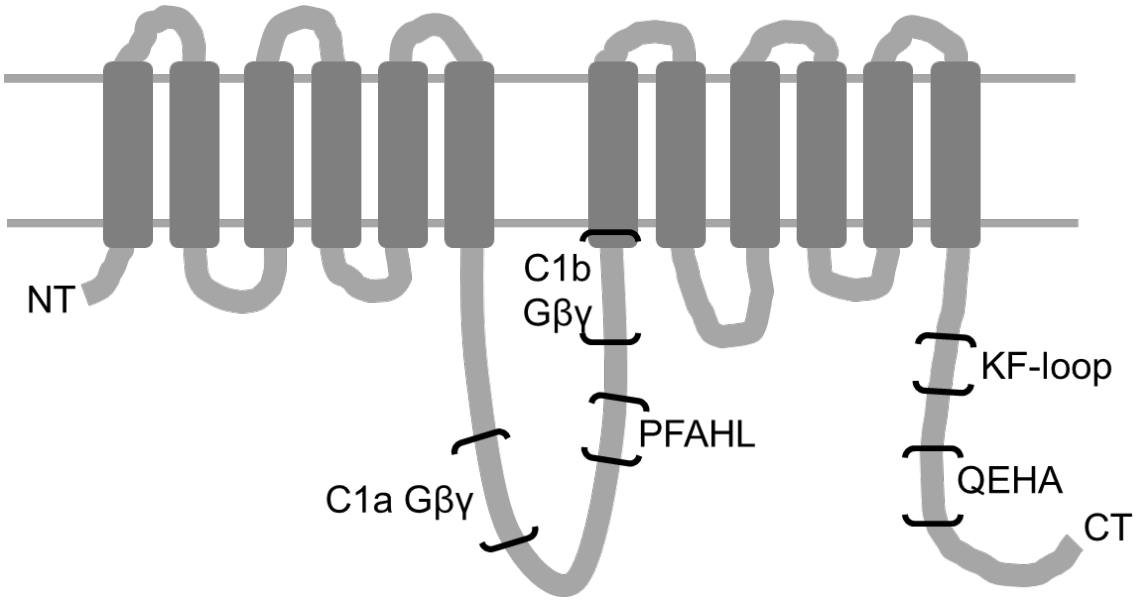

Figure 1.6 Gβγ subunit interaction sites on adenylyl cyclase 2.

Novel regulation of AC7 by Gα12/13 has also been reported. Initially, a synergistic cAMP response to isoproterenol and sphingosine-1-phosphate in RAW 264.7 macrophages was found to be mediated by the G13 pathway (Jiang et al., 2007). Subsequent research identified AC7 as the specific AC mediator of the synergistic response (i.e., AC2 did not mediate the synergistic response) and it was also shown to be dependent on Gα12/13 subunits (Jiang et al., 2008). Furthermore, the generation of a series of AC7/AC2 chimeras identified the C1a and C1b domains of AC7 to be important for the synergistic response (Jiang et al., 2013b).

1.4.2.3 Group III ACs

The group III ACs (AC5 and AC6) are known as the Ca^{2+}-inhibited group of ACs, as they are inhibited by submicromolar concentrations of free Ca^{2+}. Analogous to the modulation observed for group I ACs, it is hypothesized that CCE is the mechanism by which AC5 and AC6 are physiologically modulated by free Ca^{2+}, including in response to Gq-mediated receptor activation (Fagan et al., 2000; Willoughby and Cooper, 2007). In contrast to the inhibition of group III ACs by Ca^{2+}, Gq-coupled receptor activation in cardiac fibroblasts and gastric smooth muscle (tissues that express both AC5 and AC6) provides enhanced downstream AC signaling (Beazely and Watts, 2006; Meszaros et al., 2000; Ostrom et al., 2003). However, Gq-mediated signaling observed in stably transfected HEK293 cells indicates that Gq-mediated signaling enhances AC6,

but not AC5, suggesting a possibility for selective modulation of AC6 (Beazely et al., 2005).

AC5 and AC6 are robustly stimulated by Gαs subunits and inhibited by Gαi subunits (Chen-Goodspeed et al., 2005). Though *in vitro* studies suggest that group III ACs are insensitive to Gαo (Taussig et al., 1993a; Taussig et al., 1994), a number of intact cell and studies of cAMP signaling in tissues that express Group III ACs and Gαo suggest that receptor-mediated Gαo signaling can modulate AC5 and AC6 activity (for review, see (Beazely and Watts, 2006)). Some evidence exists for the modulation of group III ACs by Gβγ subunits. For example, overexpression studies in cells suggest that Gβγ expression inhibits AC5 and AC6 (Bayewitch et al., 1998). However, a more recent study suggests that Gβγ subunits bind directly to the N-terminus of AC5 and AC6 as part of a GαsGβγ heterotrimer and enhance Gαs-stimulated activity (Gao et al., 2007; Sadana et al., 2009). The differences in the reports of Gβγ modulation of AC5 and AC6 may be attributed to differences in the experimental methodology.

Protein kinases are also thought to modulate AC5 and AC6 activity. For example, PKA phosphorylation of AC5 and AC6 has been observed and provides feedback inhibition for these isoforms (Bauman et al., 2006; Beazely et al., 2005; Iwami et al., 1995). Furthermore, PKA-mediated inhibition of AC6 occurs via a single phosphorylation site in the C1 domain (S674) (Chen et al., 1997), and the corresponding residue in AC5 also mediates PKA-dependent inhibition (Bauman et al., 2006).

The effects of PKC activity on the modulation of group III ACs appear to be complex. AC5 activity is thought to be enhanced by PKC activity, as both *in vitro* and cell-based assays demonstrate stimulation of AC5 (Kawabe et al., 1996; Kawabe et al., 1994). The effects of PKC activity on AC6, however, are difficult to interpret because a range of modulation has been observed. For example, early studies of phorbol ester treatment on recombinant AC6 transiently expressed in HEK293 cells suggest only modest (Jacobowitz et al., 1993) or no significant stimulation of cAMP accumulation (Yoshimura and Cooper, 1993). However, PKC has been demonstrated to inhibit AC6 activity in *in vitro* membrane experiments (Lai et al., 1999; Lin et al., 2002). In contrast, phorbol ester treatment potentiated forskolin- and Gαs-mediated cAMP in HEK293 cells stably expressing AC6 (Beazely et al., 2005). Furthermore, phorbol ester treatment similarly potentiated drug-stimulated cAMP in two cell models thought to abundantly express AC6 (Chinese hamster ovary cells and Cath.a differentiated cells) (Beazely et al., 2005; Johnston et al., 2002; Varga et al., 1998). The contrasting observations surrounding PKC modulation of AC6 may reflect differential modulation by PKC isoforms and/or differences in the specific experimental approaches that were utilized.

1.4.2.4 Group IV AC

AC9 is the lone member of group IV and is most recognized for its relative insensitivity to stimulation by forskolin. The forskolin insensitivity is attributed to a

single residue in the C2 domain (Y1021) because mutation of this residue to leucine functionally restored sensitivity to forskolin (Yan et al., 1998). Early studies suggest that AC9 is stimulated by Gαs (a property common to every AC isoform) (Hacker et al., 1998), but distinctly inhibited by Ca^{2+}/calcineurin (Paterson et al., 1995). Gi/o-coupled receptor activation inhibits Gαs-stimulated AC9 activity in a pertussis-toxin dependent manner, suggesting that AC9 is also sensitive to inhibition by Gαi subunits (Cumbay and Watts, 2004). However, Gβγ subunits may also contribute to AC9 inhibition because potentiation of Gαs-stimulated cAMP activity in cells stably expressing AC9 was observed upon sequestration of Gβγ subunits (via Gαt expression) (Cumbay and Watts, 2005). In addition to G protein modulation, AC9 appears to be susceptible to modulation by protein kinases. For example, phorbol ester treatment inhibits Gαs-stimulated AC9 activity in a PKC isoform-dependent manner, as the PKC inhibitor bisindolylmaleimide I was able to block phorbol ester-mediated inhibition, but Gö6976 (a PKCα and –β selective inhibitor) had no effect (Cumbay and Watts, 2004). Interestingly, studies of Gq-mediated AC9 modulation identified a Ca^{2+}/calmodulin/CaMKII-mediated potentiation of Gαs-stimulated AC9 activity that is PKC-independent (Cumbay and Watts, 2005). These studies suggest that AC9 is complexly modulated by multiple arms of Ca^{2+} and kinase signaling pathways.

1.4.3 Adenylyl cyclase localization: Tissue distribution, compartmentalization, and signalosomes

The specificity of AC signaling can be further attributed to the tissue-specificity and subcellular localization of AC isoforms and by their protein-protein interactions within these locations. The AC isoforms are thought to display unique tissue distribution profiles. It is notable that most AC expression studies are inferred from mRNA measurements, as relatively low AC expression and the lack of specific antibodies have hindered the study of AC isoform protein expression (Hanoune and Defer, 2001). Table 1.1 summarizes the unique tissue expression of AC isoforms. These expression patterns, together with the AC isoform-dependent regulatory properties discussed above, suggest that the concurrent expression of AC isoforms and other signaling molecules contribute to distinct, tissue-specific AC-mediated signaling pathways.

Table 1.1 Adenylyl cyclase tissue distribution

AC isoform	Tissue Distribution
AC1	Brain, adrenal gland
AC2	Brain, skeletal muscle, lung, heart
AC3	Brain, olfactory epithelium
AC4	Brain, heart, kidney, liver, lung
AC5	Brain, heart, kidney, liver, lung
AC6	Widespread
AC7	Brain, platelets, widespread
AC8	Brain, lung
AC9	Brain, skeletal muscle, widespread

AC isoforms are also thought to selectively reside in specific membrane microdomains (Ostrom et al., 2012; Ostrom and Insel, 2004). The segregation of AC isoforms into membrane microdomains such as lipid rafts may contribute to specificity observed for cAMP signaling. Lipid rafts are sphingolipid and cholesterol rich portions of the plasma membrane (Simons and Ikonen, 1997) and it is thought that AC isoforms differentially localize to either lipid raft or non-lipid raft domains of the membrane (Willoughby and Cooper, 2007). Specifically, the Ca^{2+}-modulated ACs (AC1, AC3, AC5, AC6, and AC8) are thought to reside in lipid rafts, whereas the Ca^{2+}-insensitive ACs (AC2, AC4, AC7, and AC9) localize to non-lipid raft domains (Ostrom and Insel, 2004; Willoughby and Cooper, 2007). Furthermore, many signaling molecules that are associated with AC signaling including receptors, G proteins, and other effectors also reside with ACs in specific membrane microdomains (Allen et al., 2007; Patel et al., 2008). A relevant example of AC signaling specificity imparted by lipid raft organization has been observed in human bronchial smooth muscle cells. It has been demonstrated that β adrenergic receptor-mediated signaling specifically activates AC6, whereas EP_2 prostanoid receptor activation specifically modulates AC2/AC4 signaling in this cell type (Bogard et al., 2012; Bogard et al., 2011). Though all ACs are sensitive to stimulation by Gαs (Iyengar, 1993), and both β adrenergic receptors and EP_2 prostanoid receptors are Gs-coupled receptors, AC-isoform-specific signaling was observed. It is particularly notable that the selective signaling is consistent with the membrane microdomain organization of receptors and AC isoforms in human bronchial smooth muscle cells (AC6 and β

adrenergic receptors are in lipid rafts, whereas AC2/AC4 and EP_2 prostanoid receptors are in non-raft membrane domains) (Bogard et al., 2011). Similar specific receptor-AC signaling has also been observed according to localization in membrane microdomains of other tissues including aortic smooth muscle cells and cardiac myocytes (Ostrom et al., 2001; Ostrom et al., 2002; Ostrom et al., 2000). Thus, it is apparent that organization of signaling molecules into membrane microdomains contributes to signaling efficiency and specificity by concentrating specific receptor-AC groups in close proximity.

Lipid raft structures also appear to be important for CCE-modulation of ACs (Willoughby and Cooper, 2007). Disruption of lipid rafts by sequestration of cholesterol is known to disrupt the CCE modulation of AC6 (Fagan et al., 2000) and AC8 (Smith et al., 2002). It is also thought that CCE machinery is localized to lipid rafts, suggesting that CCE-modulated AC signaling is dependent on membrane organization by lipid rafts (Martin et al., 2009; Pani et al., 2008). It is clear that AC signaling modulation (and specificity) is influenced by the localization of ACs and additional signaling molecules that modulate AC function within specific membrane microdomains.

Cyclic AMP signaling specificity is also controlled by compartmentalization of AC-containing multi-protein signalosomes (Dessauer, 2009). For example, the AKAP family of proteins contributes to the signaling specificity that is observed with cAMP signaling by enabling close proximity of signaling components via signalosomes (Taylor et al., 2012; Welch et al., 2010). Over 50 AKAPs have been identified and each AKAP has a common PKA regulatory subunit

interaction motif, but are otherwise divergent (Welch et al., 2010). AKAPs are localized to various subcellular areas and function to anchor PKA to specific substrates (Dessauer, 2009). Interestingly, several of the AKAPs that localize to the plasma membrane selectively associate with AC isoforms (Dessauer, 2009). In some cases, AKAPs are thought to be components of a feedback loop for ACs that are directly modulated by PKA. For example, AKAP79/150 directly interacts with AC5/AC6 and also scaffolds PKA (Bauman et al., 2006). Expression of AKAP79/150 functionally contributes to the modulation of AC5 by promoting accelerated decay of Gs-coupled receptor-mediated cAMP responses via PKA phosphorylation (Bauman et al., 2006). These observations suggest that AKAP79/150 is part of a negative feedback loop to attenuate AC5/AC6 activation (consistent with Group III ACs being inhibited by PKA phosphorylation). Similar observations were also reported for PKA-dependent modulation of AC5 signaling that is mediated by mAKAPβ in cardiac myocytes (Kapiloff et al., 2009).

In addition to PKA scaffolding, perhaps signaling efficiency is also attained by additional scaffolding functions of AKAPs. It is known that AKAPs interact with both upstream modulators and downstream effectors of AC (protein kinases, G protein-coupled receptors, PDEs, and Epacs) (Bauman et al., 2007; Dodge-Kafka and Kapiloff, 2006; Dodge-Kafka et al., 2005). This is especially interesting in light of AKAP-mediated AC modulation that is thought to be independent of PKA function. For example, the AKAP Yotiao directly interacts with AC2 and functionally inhibits AC2-mediated cAMP signaling (Piggott et al., 2008), but AC2 is not thought to be modulated by PKA. The mechanism of

Yotiao modulation of AC2 is unclear, but possibilities include anchoring of PDEs in proximity of AC2 or by direct allosteric modulation (Dessauer, 2009). Together, these studies suggest that AKAPs are diverse modulators of AC signaling, but an appreciation of the underlying mechanisms of AKAP-mediated AC modulation requires further study. A growing number of AC-interacting proteins that modulate cAMP signaling (Sadana and Dessauer, 2009; Wang et al., 2009) support the hypothesis that signalosome-mediated AC modulation is an important contributor to cAMP signaling specificity and may even be crucial components of the mechanisms that control cAMP-mediated signaling diversity.

1.5 Heterologous sensitization of adenylyl cyclase

Acute activation of Gi/o-coupled receptors generally leads to attenuation of cAMP accumulation by inhibition of AC, but prolonged stimulation of Gi/o-coupled receptors results in heightened sensitivity to subsequent stimulatory input to AC (Watts and Neve, 2005). This phenomenon, known as heterologous sensitization of AC (also known as superactivation, cAMP overshoot, and supersensitization), was first observed in Dr. Marshall Nirenberg's laboratory in 1975 and was hypothesized to be a molecular form of adaptation that contributes to opiate dependence and tolerance (Sharma et al., 1975). Since the observation of δ opioid receptor-mediated AC sensitization in Nirenberg's lab, persistent activation of several additional types of Gi/o-coupled receptors

including D2 dopamine, 5HT1A serotonin, μ opioid, and CB1 cannabinoid receptors have also been found to mediate sensitization, suggestive of a signaling adaptation that is common for nearly all Gi/o-coupled receptors (Watts and Neve, 2005). Despite the prevalence of AC sensitization and the study of this adaptive response since 1975, an understanding of the signaling steps that link the initial Gi/o signaling event to the enhancement of AC responsiveness remains elusive.

That sensitization occurs following prolonged Gi/o-coupled receptor activation and is inhibited by pertussis toxin suggests that G protein subunits from Gi/o heterotrimers are central to the development of sensitization (Watts, 2002). Subsequent studies utilized pertussis-toxin insensitive Gα subunits as tools to investigate the specific roles of individual Gαi/o subunit isoforms in heterologous sensitization of AC. Pertussis toxin-insensitive Gαo (but not pertussis toxin-insensitive $G\alpha i_{1,\ 2,\ or\ 3}$) was capable of supporting D2 dopamine receptor-induced AC sensitization, but with a reduced response as compared to that observed in the absence of pertussis toxin treatment in NS20Y cells stably expressing the D2 dopamine receptor (Watts et al., 1998). Consistent with these observations, pertussis toxin-insensitive $G\alpha i_{1,\ 2,\ or\ 3}$ subunits are also unable to support sensitization in response to μ or κ opioid receptor-activation (Tso and Wong, 2000; Tso and Wong, 2001). In contrast, another study reported that individual pertussis toxin-insensitive $G\alpha i_{1,2,3,}$ and Gαo subunits are able to support μ opioid receptor-mediated sensitization of AC (but with a reduced level of sensitization) in both C6 glioma and HEK293T cells and that the level of

sensitization observed in these cell types is dependent on the expression levels of the pertussis toxin-insensitive G protein subunits (Clark and Traynor, 2006). Furthermore, pertussis toxin-insensitive $G\alpha i_1$ partially recovers δ opioid receptor-induced sensitization (but pertussis toxin-insensitive $G\alpha i_3$ and $G\alpha i_{2/z}$ have no effect) (Tso and Wong, 2000; Tso and Wong, 2001). The attenuated sensitization responses that are mediated by individual pertussis toxin-insensitive Gαi/o subunits suggest the possibility that multiple Gαi/o subunit isoforms simultaneously contribute to the development of the full sensitization response.

Pertussis toxin treatment also inhibits the rearrangement/release of Gβγ subunits, thus implicating Gβγ subunit signaling in the development of heterologous sensitization of AC. The most direct evidence for Gβγ subunits in sensitization has been obtained with the Gβγ subunit-sequestering proteins Gαt and the C-terminus of G protein-coupled receptor kinase 2 (GRK2-ct or βARK-ct). Multiple studies demonstrate a consensus that expression of Gβγ subunit sequestering tools provides inhibition of heterologous sensitization of AC in response to persistent activation of several G protein-coupled receptors (AvidorReiss et al., 1996; Nguyen and Watts, 2005; Rhee et al., 2000; Rubenzik et al., 2001; Thomas and Hoffman, 1996). Given that Gβγ subunits differentially influence the direction of AC modulation in an AC isoform-dependent manner (see AC isoform regulatory properties), it is unlikely that the development of heterologous sensitization is modulated by direct interactions of Gβγ with AC isoforms. Thus, it is intriguing that Gβγ subunits are becoming more recognized for modulation of diverse signaling pathways (Khan et al., 2013; Smrcka, 2008),

suggesting that Gβγ subunits are components of complex signaling pathways that may lead to the development of AC sensitization.

Protein kinases modulate the activity of AC isoforms (see the regulatory properties of AC section). It is therefore possible that prolonged Gi/o-coupled receptor activation leads to the downstream modulation of protein kinases that are able to enhance the activity of ACs, resulting in sensitization of AC. Consistent with this hypothesis is that Gαi and Gβγ signaling are known to modulate protein kinases that include PKC, PKA, and Raf-1 (Watts and Neve, 2005). For example, PKC enhances the activity of several AC isoforms (i.e., AC1, AC2, AC5, AC6, and AC7) and heterologous sensitization has been observed in cells that express AC2 and AC5, suggesting a role for PKC in the development of AC sensitization. Furthermore, enhanced PKC-mediated phosphorylation of ACs (group II ACs) was observed in ileum longitudinal muscle myenteric plexus preparations obtained from chronic morphine-treated guinea pigs (Chakrabarti et al., 1998).

Several studies also implicate Raf-1 kinase in heterologous sensitization. For example, pharmacological inhibition of Raf-1 kinase in Chinese hamster ovary (CHO) cells decreased cAMP signaling, suggesting that Raf-1 can stimulate AC6 activity (endogenously expressed) (Tan et al., 2001; Varga et al., 2003; Varga et al., 1998). Consistent with this observation, studies also suggest that Raf-1 kinase inhibition attenuates sensitization of AC in CHO cells (Beazely et al., 2005; Varga et al., 2002; Varga et al., 2003). Furthermore, studies suggest that Raf-1 can enhance AC activity upon modulation by PKC and

receptor tyrosine kinases (Beazely et al., 2005; Chen et al., 1995b; Tan et al., 2001; Varga et al., 2003).

Protein kinase A may also have a role in heterologous sensitization of ACs. For example, it is known that PKA phosphorylation inhibits AC5 and AC6 activity, suggesting that inhibitory modulation of PKA may enhance the catalytic activity of these isoforms. Consistent with this hypothesis, activators of PKA attenuated sensitization, whereas inhibitors of PKA induced sensitization in a cell line that endogenously expresses AC6 (Johnston et al., 2002). Additional studies also support a role for PKA in sensitization via the use of a PKA-deficient cell line (Thomas and Hoffman, 1989) and activators of PKA in DDT1-MF2 cells (Port et al., 1992). Contrary to these studies, several approaches also suggest that PKA is not essential for heterologous sensitization of AC (Avidorreiss et al., 1995; Watts and Neve, 1996; Watts et al., 1999). These differences may be reflective of the complement of ACs and other signaling molecules that are present in each given system and demonstrate the complexity associated with differential modulation of AC isoforms by protein kinases.

1.6 Adenylyl cyclase isoforms in physiology and disease

AC isoforms are implicated in a variety of physiological processes and disease states in both the central nervous system and the periphery. Much of our current understanding of the contribution of individual AC isoforms to

biological processes is from knockout and transgenic animal studies and is summarized below.

1.6.1 Adenylyl cyclase function in peripheral tissues

Knockout and transgenic overexpression models implicate both AC5 and AC6 in cardiac function and heart failure. Specifically, AC5 deletion impairs sympathetic, parasympathetic, and Ca^{2+}-mediated regulation of left ventricular function (Okumura et al., 2003a; Tang et al., 2006). However, cardiac-directed overexpression of AC5 enhances heart rate and fractional shortening, but does not differ from wild type mice in response to β adrenergic receptor stimulation (Tepe et al., 1999). Interestingly, deletion of AC6 has no significant effect on the basal left ventricular function, but disrupts cAMP signaling and Ca^{2+} handling and reveals a role for AC6 in β adrenergic receptor-mediated left ventricular function (Tang et al., 2008). Deletion of AC5 is protective against increased apoptosis and reduces the left ventricular ejection fraction associated with heart failure in response to pressure overload induced by thoracic aortic banding (Okumura et al., 2003b). Targeted overexpression of either AC5 or AC6 displays protective effects in cardiac function in response to Gq-expression induced cardiomyopathy (Roth et al., 1999; Tepe and Liggett, 1999), but only AC6 overexpression reduces hypertrophy and enhances survival (Roth et al., 2002). Interestingly, subsequent studies further suggest beneficial effects of AC6 expression for cardiac function (for review, see (Gao and Hammond, 2011)) and that many of

these effects are independent of the cAMP-generating catalytic function of AC6 (Gao and Hammond, 2011; Gao et al., 2011). Further stratifying the biological functions of AC5 and AC6, $AC5^{-/-}$ mice show protective effects against age-induced cardiomyopathy and display increased life-span associated with additional protective effects against age-related oxidative stress, apoptosis, and loss of bone quality (Yan et al., 2007). In summary, AC5 and AC6 demonstrate overlapping, but distinct functions in cardiac function and models of heart failure and AC5 is implicated in pathophysiological processes associated with aging.

Immune responses also appear to be mediated by at least one AC isoform. For example, AC7 has recently been implicated in distinct types of immune responses. Specifically, mice with AC7-deficient immune systems are sensitive to endotoxic shock (via lipopolysaccharide (LPS) injection) as measured by survival following a semi-lethal dose of LPS (Duan et al., 2010). Biochemical analysis revealed blunted cAMP responses and enhanced pro-inflammatory cytokine production in macrophages from the AC7-deficient mice, suggesting that AC7-mediated cAMP signaling may underlie immune responses to bacterial infection (Duan et al., 2010). The AC7-deficient mice also display an overall reduction of cAMP signaling in T and B cells, and altered antibody responses (Duan et al., 2010). Interestingly, it has also been recently proposed that certain pathogens may evade host immune responses by enhancing AC7-mediated signaling in macrophages, thereby deceiving the system into reduced pro-inflammatory cytokine responses (Jiang et al., 2013a). These data suggest that AC7-mediated cAMP signaling contributes to distinct types of immune responses

and is potentially modulated by pathogens to evade host immune responses (Duan et al., 2010; Jiang et al., 2013a).

1.6.2 Adenylyl cyclase function in the central nervous system

Several AC isoforms are implicated in distinct types of pain and analgesia. AC5 is thought to mediate both acute and chronic pain responses. Specifically, $AC5^{-/-}$ mice display reduced behavioral responses to acute pain elicited by thermal and mechanical stimuli, inflammatory stimuli, and visceral nociceptive stimuli (both inflammatory and non-inflammatory) (Kim et al., 2007). Furthermore, $AC5^{-/-}$ mice show attenuated mechanical and thermal allodynia in two separate neuropathic pain models (the L5 spinal nerve ligation model and a tail nerve injury model) (Kim et al., 2007). Interestingly, the Ca^{2+}/calmodulin-stimulated ACs, AC1 and AC8 are also implicated in chronic pain, but not acute pain responses. $AC1^{-/-}$, $AC8^{-/-}$, and $AC1^{-/-}/AC8^{-/-}$ mice are not different than wild type mice in acute pain response to noxious thermal, mechanical, and inflammatory stimuli (Vadakkan et al., 2006; Wei et al., 2002). However, $AC1^{-/-}$ and $AC1^{-/-}/AC8^{-/-}$ mice display reduced responses to chronic inflammatory stimuli (injection of formalin and complete Freund's adjuvant injection) (Vadakkan et al., 2006; Wei et al., 2002). Furthermore, $AC1^{-/-}/AC8^{-/-}$ mice show attenuated behavioral responses to nerve injury (Wei et al., 2002).

Multiple AC isoforms have also been distinctly associated with morphine action. AC5 is thought to mediate the behavioral effects of morphine because

$AC5^{-/-}$ mice lost morphine-induced analgesia (latency for response to acute thermal stimuli), locomotor activity, reward, dependence, and behavioral signs of withdrawal (Kim et al., 2006). On the other hand, mice with genetic deletion of Ca^{2+}/calmodulin-stimulated ACs (AC1 and AC8) retain short-term morphine-induced analgesia, but display reduced tolerance and withdrawal-associated behaviors (Li et al., 2006; Zachariou et al., 2008). Interestingly, transgenic mice overexpressing AC7 in the CNS demonstrate enhanced sensitivity to the acute analgesic effects of morphine (measured by paw-lick latency in the hot plate test) and show more rapid tolerance to morphine treatment, but no difference in withdrawal-associated behaviors as compared to wild type animals (Yoshimura et al., 2000).

The Ca^{2+}/calmodulin-stimulated ACs are implicated in important CNS-related biological processes that are associated with aging. For example, the Ca^{2+}/calmodulin-stimulated ACs are thought to have redundant roles in long term memory, as $AC1^{-/-}/AC8^{-/-}$ mice are deficient in late phase long-term potentiation and long term memory, but genetic deletion of AC1 or AC8 individually has no effect on these measures (Wong et al., 1999). The Ca^{2+}/calmodulin-stimulated ACs are also implicated in neurodegenerative processes including excitotoxicity. Excitotoxicity is neuronal damage/death that results from overstimulation of NMDA receptors by glutamate. The excessive stimulation of NMDA receptors results in a Ca^{2+} overload in neurons, resulting in cell death. It is thought that excitotoxicity contributes to the acceleration of neurodegenerative processes. AC1 is associated with excitotoxicity because primary cortical neurons from

$AC1^{-/-}$ mice are resistant to glutamate- and NMDA-induced excitotoxicity and $AC1^{-/-}$ mice have attenuated neuronal cell death in response to intracortical injection of NMDA (Wang et al., 2007a). Ca^{2+}-stimulated ACs are further implicated in neurodegeneration in neonatal mice. Specifically, ethanol, the GABAa receptor modulator phenobarbital, and the NMDA receptor antagonist MK801 each enhance neurodegeneration in neonatal $AC1^{-/-}/AC8^{-/-}$ mice. Furthermore, $AC1^{-/-}$ or $AC8^{-/-}$ mice display similar neurodegenerative effects (Maas et al., 2005). In summary, several AC isoforms contribute to biological processes in the central nervous system including pain, analgesia, learning and memory, and neurodegeneration.

1.7 Small molecule modulation of adenylyl cyclase

It is clear that AC isoforms are implicated in several disease states and it is hypothesized that AC isoform-selective small molecules could be utilized together with knockout and transgenic animal models to investigate the physiological roles of AC isoforms and validate their therapeutic potential (Pavan et al., 2009; Pierre et al., 2009). However, small molecule AC modulators are generally non-selective and/or display low potency for AC modulation (Seifert et al., 2012). The current collection of AC modulators is discussed below.

Forskolin (Figure 1.7) is a labdane diterpene that was isolated from the roots of the *Coleus forskohlii* plant, and was found to be a reversible activator of

adenylyl cyclase activity in several tissues including the cerebral cortex, striatum, heart, and liver (Seamon et al., 1981). Subsequent studies with recombinant adenylyl cyclases suggest that forskolin directly binds AC and is capable of stimulating all AC isoforms with the exception of AC9 (Sadana and Dessauer, 2009; Tesmer et al., 1997). Though forskolin has been used extensively as a research tool for the study of ACs and cAMP signaling (Insel and Ostrom, 2003), several properties of forskolin are suboptimal. For example, forskolin is not water-soluble, modulates AC isoforms in a non-selective fashion, and is known to modulate other enzymes including glucose transporters (Laurenza et al., 1989). To improve upon these shortcomings, much effort has been directed toward the synthesis and pharmacological characterization of forskolin analogs. For example, NKH477 was identified as a water-soluble forskolin analog that has enhanced potency for AC5 as compared to AC3 and AC2 (Toya et al., 1998). More recent studies identified forskolin derivatives that display a range of modulatory properties, including inactivity, partial activation, enhanced efficacy for AC isoforms, and inhibition of AC isoforms (Onda et al., 2001; Pinto et al., 2008). Notably, BODIPY-conjugated forskolin displays potent inhibition of AC2, while partially activating AC1 and AC5 (Pinto et al., 2008). Additional chemical modification of forskolin may yield derivatives with novel modulatory properties or more favorable AC isoform-selectivity profiles, but studies to date have only provided marginal improvements.

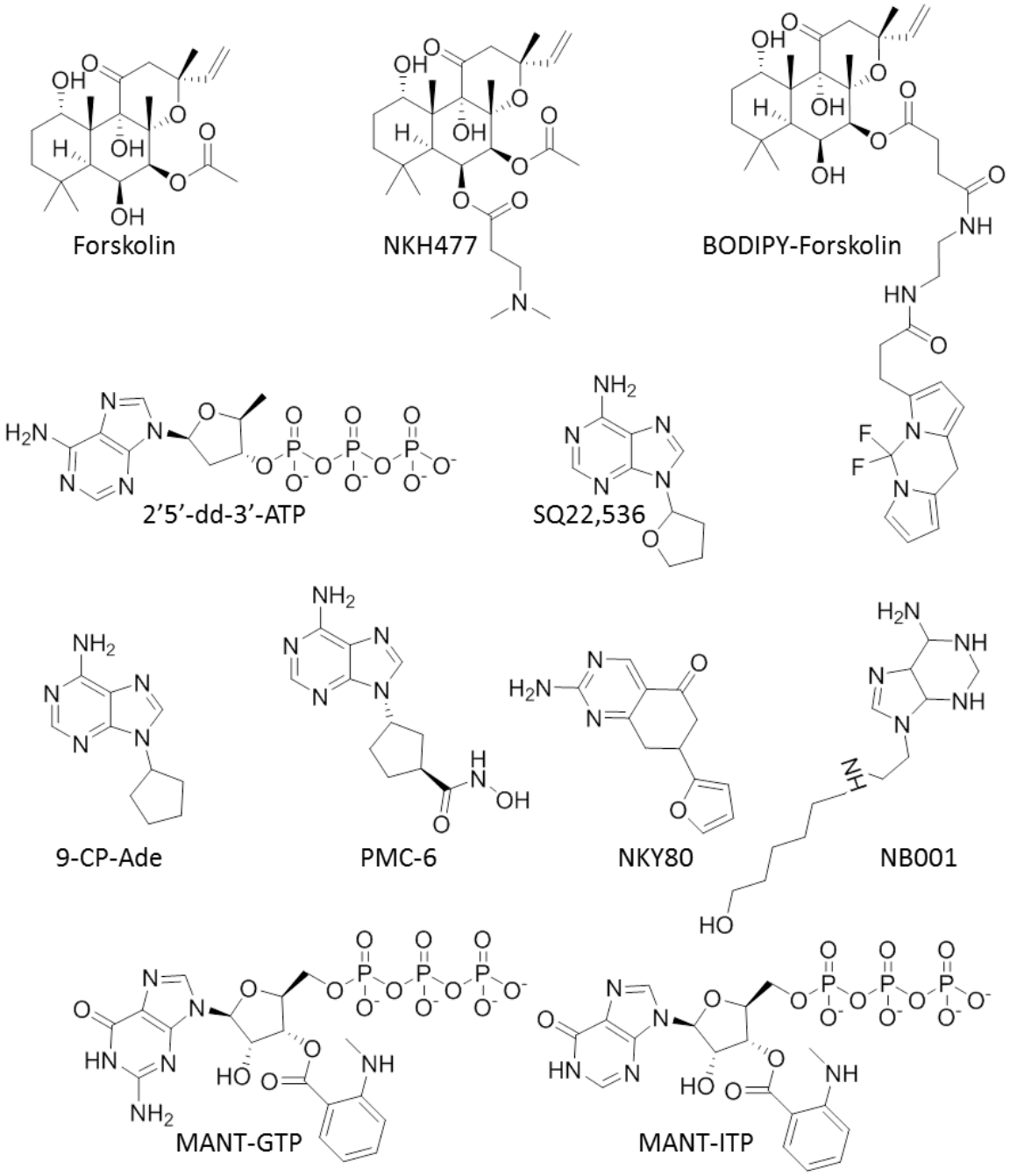

Figure 1.7 Chemical structures of small molecule adenylyl cyclase modulators.

Early approaches to the development of adenylyl cyclase inhibitors focused on compounds that are nucleotides that contain an adenine (or purine) ring, known as the “p-site” inhibitors (Dessauer et al., 1999). The p-site inhibitors are generally non-selective for inhibition of adenylyl cyclase isoforms (Johnson et al., 1997) and bind to the active site in the presence of pyrophosphate (Tesmer et al., 2000), but non-competitively with respect to ATP (Dessauer and Gilman, 1997; Tesmer et al., 2000). It is clear that the nucleotide phosphate groups confer potency for inhibition of ACs, as adenine nucleoside 3’-polyphosphate ligands are among the most potent p-site inhibitors (Johnson et al., 1997). However, the polyphosphate groups are expected to be poorly membrane-permeable. Though p-site ligands are generally non-selective, ribose-modified p-site ligands such as SQ22,536 and 9-CP-Ade display some degree of AC isoform selectivity *in vitro* (i.e., both compounds have similar potency values for inhibition of AC1, AC6, and AC8, but little or no activity for inhibition of AC2), but are relatively less potent than the adenine 3’-polyphosphate p-site ligands (Johnson et al., 1997). The p-site inhibitors identified to date appear to have a trade-off between potency and AC isoform selectivity. Furthermore, p-site inhibitors may have off-target effects such as inhibition of DNA replication or purine metabolism due to their adenine rings (Seifert et al., 2012).

Given concerns about an intact adenine ring facilitating off-target effects, a virtual screen was conducted for compounds that retained the pharmacophore of a ribose-substituted p-site inhibitor for AC, but without an adenine ring (Onda et al., 2001). This screen yielded the second generation p-site inhibitor, NKY80

(Onda et al., 2001). Though NKY80 displays selectivity for AC5 over AC2 and AC3, and binds non-competitively with ATP and forskolin, the compound generally lacks potency for inhibition of AC (Onda et al., 2001).

An additional strategy for the development of more potent and/or selective inhibitors of AC involved the fusion of a metal-coordinating hydroxamic acid moiety to an adenine ring to exploit the requirement of metal ions for AC catalytic activity (Levy et al., 2003; Levy et al., 2002a; Levy et al., 2002b; Tesmer et al., 1999). In *in vitro* Sf9 cell membrane assays, PMC-6 emerged as a selective AC5 inhibitor (over AC2 and AC3), and the most potent among the PMC class of inhibitors (Iwatsubo et al., 2004). Furthermore, PMC-6 has activity for inhibition of isoproterenol-stimulated cAMP in intact cardiac myocytes, where it also displays efficacy for inhibition of apoptosis (Iwatsubo et al., 2004), suggesting possible therapeutic utility.

More recent studies have identified NB001 as a potent inhibitor of AC type 1 in intact cell assays (Wang et al., 2011). Interestingly, in agreement with AC1 knockout mouse studies, inhibition of AC1 activity with NB001 has analgesic effects in animal models of neuropathic and inflammatory pain (Vadakkan et al., 2006; Wang et al., 2011). These studies suggest that targeting AC1 has analgesic utility, but the activity of NB001 in *in vitro* AC assays and an understanding of the structure-activity relationship surrounding NB001 remain to be reported.

2’(3’)-O-N-Methylanthraniloyl (MANT)-substituted fluorescent nucleotides were found to be a novel and potent class of competitive AC inhibitors (Gille and

Seifert, 2003). Subsequent crystallography studies with MANT-GTP suggest that the MANT moiety binds in a hydrophobic patch at the C1/C2 interface and prevents a necessary rotation that is required for AC catalytic activity (Mou et al., 2005; Wang et al., 2007b). Furthermore, a series AC isoform-selectivity and structure-activity relationship studies on the MANT class of compounds revealed a general preference for inhibition of AC5 and AC6 over other AC isoforms and that the catalytic site can accommodate nucleotides including adenine, guanine, hypoxanthine, and uracil (Gille et al., 2004; Goettle et al., 2009; Huebner et al., 2011; Mou et al., 2006; Pinto et al., 2011; Suryanarayana et al., 2009). Consistent with the latter observation, MANT-ITP was identified as the most potent AC inhibitor from this class of compounds. Though the MANT nucleotides are generally more potent than the p-site inhibitors for inhibition of AC *in vitro*, these compounds are also limited by poor membrane-permeability (Seifert et al., 2012). Thus, these studies are most useful for the structural information that they provide surrounding the binding of ligands in the catalytic site of ACs.

Efforts to develop potent and AC isoform-selective small molecule modulators have only modestly contributed to the current understanding of AC targeting and several limitations surrounding specificity, AC isoform-selectivity, and cell permeability remain. Furthermore, the current repertoire of AC modulators has not been comprehensively studied with respect to each of the nine membrane-bound AC isoforms in both *in vitro* and intact cell models, making it difficult to fully assess the isoform-selectivity of these compounds (Seifert et al., 2012). The discovery and development of novel small molecule modulators of

AC that are potent and isoform-selective is expected to facilitate the *in vivo* study of individual AC isoforms and allow for the full evaluation of AC isoforms as potential therapeutic targets.

1.8 Scope of the work

The goal of the work reported here is to investigate the modulation of adenylyl cyclase type 2 signaling within the context of the following three specific aims:

1.) To test the hypothesis that AGS3 expression influences D_{2L} dopamine receptor-mediated cAMP signaling mediated via AC1 and AC2. This specific aim was designed to investigate the effects of expression of the G protein regulator, AGS3, on recombinant AC1 and AC2 in response to both acute and persistent activation of the D_{2L} dopamine receptor in HEK293 cells. The differential modulation of AC1 and AC2 by G protein subunits offers unique properties to study the effects of AGS3 expression on G protein-coupled receptor signaling.

2.) To test the hypothesis that D_{2L} dopamine receptor-mediated heterologous sensitization of AC2 is Gβγ subunit-dependent. The mechanism of D_{2L} dopamine receptor-induced heterologous sensitization of AC2 is unclear. Specific aim 2 was designed to study the role of Gβγ subunit

signaling to D_{2L} dopamine receptor-mediated sensitization of AC2 in HEK293 cells.

3.) To develop and implement a high-throughput screening paradigm for the discovery of small molecule modulators of AC2. Specific aim 3 encompassed the development of a cell-based high-throughput screening paradigm for the measurement of cellular cAMP. The assay platform was then utilized to conduct an intact-cell small molecule screen for direct AC2 inhibitors in HEK293 cells. The work in this aim was designed as an intermediate step in the overall goal to develop unbiased methods for the study of AC sensitization.

CHAPTER 2. DIFFERENTIAL EFFECTS OF AGS3 EXPRESSION ON D2L DOPAMINE RECEPTOR-MEDIATED ADENYLYL CYCLASE SIGNALING

2.1 Introduction

G protein-coupled receptors bind ligands that induce conformational changes in the receptor and facilitate the activation of G protein heterotrimers by exchange of GDP for GTP on the G protein α subunit (Gilman, 1987). Upon activation, the G protein heterotrimer undergoes a conformational change that allows Gα and Gβγ subunits to regulate effector proteins such as adenylyl cyclases (ACs) (Coleman et al., 1994; Lambright et al., 1994; Taussig et al., 1993a). The G protein signaling cycle ends by hydrolysis of GTP to GDP on the Gα subunit (Coleman et al., 1994; Mixon et al., 1995). In addition to G protein-coupled receptor modulation, the G protein activation cycle is sensitive to accessory proteins such as regulators of G protein signaling (RGS) or activator of G protein signaling (AGS) proteins (Blumer et al., 2007; Neubig and Siderovski, 2002; Sato et al., 2004). These proteins influence G protein signaling and effector activation by modulating the rate of GTP hydrolysis and the availability and/or stability of Gα or Gβγ subunits (Blumer et al., 2007; Neubig and Siderovski, 2002; Sato et al., 2004)

The activator of G protein signaling 3 (AGS3) is a G protein regulator that has been linked to adaptive behaviors involved with drugs of abuse (Bowers et al., 2008; Bowers et al., 2004; Yao et al., 2005). AGS3 was identified as a receptor-independent G protein activator and is thought to bind Gαi subunits in the GDP-bound state, thereby preventing re-association of Gα and Gβγ subunits (De Vries et al., 2000; Peterson et al., 2000; Takesono et al., 1999). The precise molecular actions of AGS3 have yet to be fully described, however it is hypothesized that AGS3 expression may specifically adjust the landscape of effector activation by modulating Gα and Gβγ subunit signaling. Previous studies examining the actions of AGS3 on G protein-coupled receptor signaling in cell-based assays suggest that the effects of AGS3-like molecules are dependent on the duration of receptor activation. For example, an AGS3 consensus peptide was shown to have no effect on the ability of D_2DRs to modulate G protein-regulated inwardly rectifying potassium (GIRK) channels acutely, but promoted functional desensitization of this response following repeated receptor activation (Webb et al., 2005). Furthermore, the actions of AGS3 on α2 adrenergic receptor regulation of cAMP signaling were explored in CHO cells, which predominantly express AC6 (Varga et al., 1998). The acute effects on α2 adrenergic receptor-mediated AC signaling were not altered, but sensitization of AC in response to persistent α2 receptor activation was attenuated by AGS3 expression (Sato et al., 2004). More recent studies in nucleus accumbens/striatal neurons have suggested that opioid receptor-induced

expression of AGS3 enhances the activity of AC5 and AC7 in a protein kinase-dependent manner (Fan et al., 2009).

The observations described above suggest a complex mode of AGS3 modulation of receptor-mediated AC signaling. The complexity is further exacerbated by the expression and signaling of multiple AC isoforms that display unique patterns of Gα and Gβγ regulation (Sunahara et al., 1996; Watts and Neve, 2005). The present study was designed to examine the effects of AGS3 on G protein-coupled receptor modulation of two recombinant ACs, AC1 and AC2. AC1 is a member of the Ca^{2+}-stimulated group of ACs and can be activated by the Ca^{2+} ionophore, A23187, and inhibited by both Gαi and Gβγ subunits (Choi et al., 1992a; Cumbay and Watts, 2001; Taussig et al., 1993b). AC2 is a member of a group of ACs that are conditionally activated by Gβγ subunits (Federman et al., 1992; Taussig et al., 1993b). AC2 is also robustly activated by protein kinase C (PKC) phosphorylation in response to phorbol ester stimulation (e.g. PMA) (Shen et al., 2012; Yoshimura and Cooper, 1993). The distinct regulatory properties of AC1 and AC2 provide important tools to selectively study the effects of AGS3 expression on G protein-coupled receptor signaling.

It is widely accepted that alterations in cAMP signaling pathways and enhanced activation of dopamine systems in the brain play central roles in the molecular adaptations associated with drug addiction (Carlezon et al., 2005; McClung and Nestler, 2003; Nestler, 2001). The dopaminergic signaling and cAMP signaling pathways are linked by dopamine receptors that modulate

adenylyl cyclases via G protein activation. There are two families of dopamine receptors, D1-like (D_1 and D_5) that couple to Gαs, and D2-like (D_2, D_3, and D_4) that couple to Gαi/o (Missale et al., 1998). We chose to study D_{2L}DR signaling based on the overlapping tissue distribution of the D_{2L}DR with AC1 and AC2 in the brain, and its well-characterized roles in AC signaling and drug abuse, where AGS3 has also been implicated (Maldonado et al., 1997; Phillips et al., 1998; Ralph et al., 1999; Visel et al., 2006; Weiner et al., 1991). HEK293 cells expressing the D_{2L}DR together with either AC1 or AC2 were used to explore cAMP signaling in the absence or presence of AGS3. Our studies revealed that AGS3 expression had modest, but significant potentiating effects on acute D_{2L}DR modulation of AC1 or AC2 activity. In contrast, AGS3 displayed differential effects on AC regulation following persistent D_{2L}DR activation. These findings, along with those reported in the literature, suggest that AGS3 modulates AC signaling in a manner that is isoform-specific and dependent on the duration of receptor activation.

2.2 Materials and methods

2.2.1 Materials

[3H]-cAMP (33 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Spiperone, (±)-quinpirole, A23187, 3-isobutyl-1-methylxanthine (IBMX), G418, Dulbecco's modified Eagle's medium (DMEM),

and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was purchased from Tocris Bioscience (Ellisville, MO). Lipofectamine 2000 and Zeocin were purchased from Invitrogen (Carlsbad, CA). FetalClone I (FCI) serum, bovine calf serum (BCS), and Earle's balanced salt solution (EBSS) were purchased from Hyclone (Logan, UT). Hygromycin B was purchased from Calbiochem (La Jolla, CA).

2.2.2 Cell culture and stable cell line generation

HEK293 cells stably expressing AC1 or AC2 were grown in DMEM supplemented with 5% FCI, 5% BCS, 1 unit/ml penicillin, 1 µg/ml streptomycin, 2.5 ng/ml amphotericin B, and either 100 µg/ml Hygromycin B (HEK-AC1) or 300 µg/ml G418 (HEK-AC2) and were maintained in a humidified incubator at 37°C with 5% CO_2. Cell lines were transfected with the dual expression vector pBudCE4 with the gene for the human D_{2L}DR driven by the CMV promoter and either rat AGS3-Venus (long splice variant), AGS3-Q/A-Venus, or no gene behind the EF-1α promoter. Several studies have utilized AGS3 fused with a fluorescent protein at the c-terminus (An et al., 2008; Oner et al., 2010; Oner et al., 2013; Vural et al., 2010) and these studies suggest that the AGS3-fluorescent fusion proteins retain similar cellular localization (An et al., 2008; Blumer et al., 2002; Pizzinat et al., 2001; Vural et al., 2010) and interaction with Gαi subunits (De Vries et al., 2000; Oner et al., 2010; Oner et al., 2013; Peterson et al., 2000) as compared to untagged AGS3. Stable transfections were carried out with

Lipofectamine 2000 according to the manufacturer's protocol. Clones were isolated by selection with Zeocin (200 µg/ml) and characterized for specific AC function by cAMP accumulation assays, D_{2L}DR expression by radioligand binding, and AGS3 expression by fluorescence microscopy.

2.2.3 Cyclic AMP accumulation assay

Cells were grown to confluency in 48-well plates and cAMP assays were performed on ice in assay buffer (EBSS containing 15 mM Na^+-HEPES, 2% BCS, and 0.02% ascorbic acid). For acute cAMP accumulation experiments, cells were stimulated at 37°C for 15 minutes in the presence of 500 µM IBMX. The stimulation buffer was decanted and cells were lysed with ice-cold 3% trichloroacetic acid. The plate was stored at 4°C for at least 1 h before cAMP quantification. For persistent receptor activation experiments, cells were grown to confluency in 48-well plates and pretreated in assay buffer with either vehicle or 1 µM (±)-quinpirole for 2 h at 37°C and 5% CO_2. Cells were washed with assay buffer three times (3 minutes each), and subsequently stimulated as described for acute cAMP accumulation assays. For desensitization assays, pretreatments were carried out as described above, followed by re-activation of the receptor as described for acute cAMP accumulation experiments. For heterologous sensitization experiments, subsequent stimulation was achieved by selective activation of AC isoforms (AC1, 10 µM A23187; AC2, 1 µM PMA) in the

presence of 500 μM IBMX and 1 μM spiperone to block residual agonist binding from the pretreatment.

2.2.4 Cyclic AMP quantification

Cyclic AMP was quantified using a competitive binding assay (Przybyla and Watts, 2010). Duplicate samples of lysate from the cAMP accumulation assay were added to reaction tubes, followed sequentially by [^{3}H]-cAMP (~1 nM final concentration), and cAMP-binding protein (~100 μg of crude bovine adrenal extract) in 500 μL of cAMP binding buffer (100 mM Tris-HCl, 100 mM NaCl, 3 mM EDTA, pH 7.4). The assay was carried out at 4°C for 2 hr and harvested by filtration through Millipore FB 96-well filter plates, and radioactivity was quantified on a TopCount NXT scintillation counter (Perkin Elmer). Cyclic AMP concentrations were estimated from a standard curve ranging from 300 pmol to 3 nmol cAMP.

2.2.5 Data analysis

GraphPad Prism software was used to generate all dose-response curves (GraphPad Software, San Diego, CA). All logIC_{50}/logEC_{50}, and maximal activation/inhibition values were calculated in GraphPad Prism using a non-linear regression and sigmoidal dose-response equation and were analyzed by unpaired *t*-tests.

2.3 Results and discussion

AGS3 interacts with the GDP-bound state of Gαi subunits, and it has been suggested that this interaction prevents the re-association of Gαi and Gβγ subunits (De Vries et al., 2000; Peterson et al., 2000). The interaction between AGS3 and Gαi-GDP may cause altered G protein signaling through effectors regulated by Gαi and Gβγ subunits. AC isoforms display differential patterns of regulation by G protein subunits (Sunahara et al., 1996; Watts and Neve, 2005). Given that AGS3 may alter signaling by Gαi and/or Gβγ subunits, and AC isoforms are differentially regulated by G protein subunits, we were interested in studying the effects of AGS3 expression on the regulation of AC isoforms following acute and persistent activation of D_{2L}DRs.

2.3.1 Characterization of HEK293 stable cell lines

To study the effects of AGS3 on D_{2L}DR-mediated signaling through AC isoforms, several HEK293 cell lines were generated and characterized (HEK-AC1-D_{2L}, HEK-AC1-D_{2L}-AGS3-Venus, HEK-AC2-D_{2L}, HEK-AC2-D_{2L}-AGS3-Venus, and HEK-AC2-D_{2L}-AGS3-Q/A-Venus). The function of the stably expressed AC isoforms was characterized by the cAMP responses to pharmacological conditions for the selective activation of either AC1 (10 μM A23187) or AC2 (1 μM PMA) (Figure 2.1A and B).

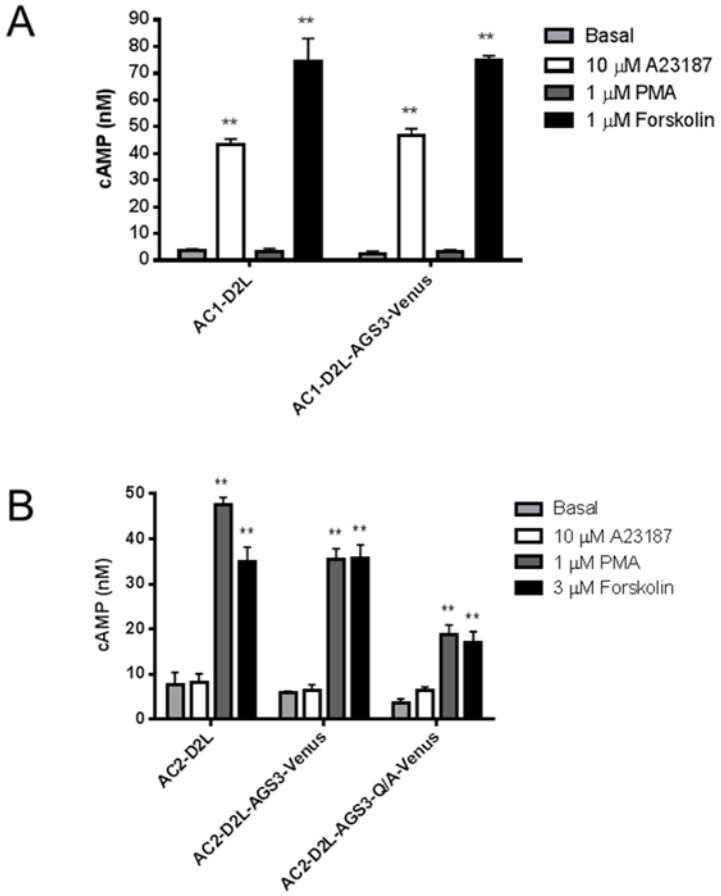

Figure 2.1 Selective activation of recombinant AC1 by the calcium ionophore, A23187 and recombinant AC2 by the phorbol ester, PMA. **A.** Cell lines expressing recombinant AC1 or **B.** recombinant AC2 were incubated under the indicated conditions and cAMP accumulation was measured using a Cisbio homogenous time-resolved fluorescent (HTRF) cAMP assay kit according to the manufacturer's protocol. Data represent the mean ± S.E.M. of three experiments. ** $p < 0.01$, compared to the basal condition within each cell line (one way ANOVA, followed by Dunnett's post hoc test).

As expected, treatment with the Ca^{2+} ionophore, A23187 significantly stimulated cAMP accumulation in cell lines expressing AC1, but had no effect in cell lines expressing AC2. On the other hand, phorbol ester treatment selectively enhanced the cAMP activity in cells expressing AC2, but had no effect in cells expressing AC1. These results suggest that the stable cell lines expressing AC1 or AC2 can be selectively activated by A23187 or PMA, respectively. The cell lines were further

characterized for the expression levels of the D_{2L}DR, as quantified by [3H]-methylspiperone saturation binding (Table 2.1). Each of the stable cell lines had similar Bmax and Kd values, suggesting that the D_{2L}DR expression levels were similar. AGS3-Venus expression was confirmed by fluorescence microscopy (Figure 2.2) and spectroscopy (Table 2.2). Together, these data suggest that cell lines that were generated are suitable for studies of the effects of AGS3 expression on D_{2L}DR mediated signaling mediated by either AC1 or AC2.

Table 2.1 D_{2L}DR expression levels in cellular models used for functional studies. [^{3}H]-Methylspiperone saturation binding assays and analysis was performed (as described in Przybyla and Watts, 2010) to measure the D_{2L}DR expression level in each cell line. Data represent the mean ± S.E.M. of four independent experiments.

Cell Line	**Bmax** *(pmol/mg protein)*	**Kd** *(nM)*
HEK-AC1-D_{2L}	2.5±0.10	0.10±0.02
HEK-AC1-D_{2L}-AGS3-Venus	4.0±0.27	0.11±0.02
HEK-AC2-D_{2L}	9.1±2.5	0.11±0.01
HEK-AC2-D_{2L}-AGS3-Venus	15±3.0	0.17±0.04
HEK-AC2-D_{2L}-AGS3-Q/A-Venus	8.9±2.1	0.10±0.01

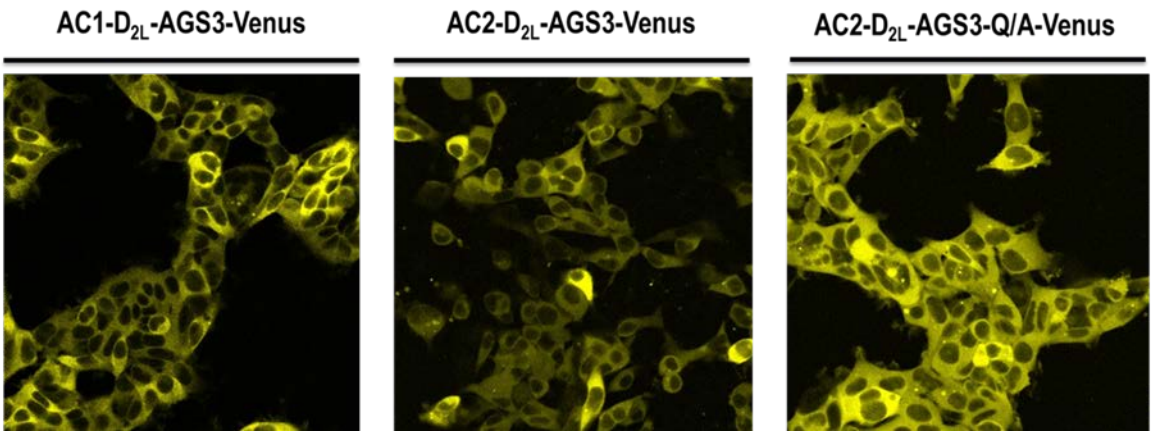

Figure 2.2 AGS3-Venus expression by confocal microscopy. Representative fields were imaged for cell lines expressing AGS3 fused to the Venus fluorescent protein at 20X magnification using a Nikon A1 confocal system when excited with the 488 nm laser and filtered for detection of Venus fluorescence (Emission: 525/50).

Table 2.2 The Venus fluorescence intensity (Ex: 485/20 nm and Em: 530/30) of cell suspensions was measured in 384-well format using a Biotek Synergy 4 plate reader. The fluorescence intensity values of HEK-AC1-D_{2L} and HEK-AC2-D_{2L} represent the background autofluorescence of the cells.

Cell Line	**Fluorescence Intensity** *(RFU)*
HEK-AC1-D_{2L}	1530±110
HEK-AC1-D_{2L}-AGS3-Venus	18900±2110
HEK-AC2-D_{2L}	1460±100
HEK-AC2-D_{2L}-AGS3-Venus	9400±2400
HEK-AC2-D_{2L}-AGS3-Q/A-Venus	33700±3270

2.3.2 Modulation of acute D_{2L}DR-mediated AC1 and AC2 signaling by AGS3 expression

Our initial studies used HEK293 cells stably expressing AC1 or AC2 and the D_{2L}DR. Recombinant AC1 can be selectively activated in HEK293 cells by Ca^{2+} using the calcium ionophore, A23187 or by capacitative calcium entry (Choi et al., 1992a; Cooper et al., 1994). To observe acute Gαi regulation, AC1 activity was selectively increased using A23187, and inhibition by the D_2DR agonist quinpirole was examined. A23187-stimulated AC1 activity was inhibited by 54±2% with a $logIC_{50}$ of -8.08±0.13 (Figure 2.3A). In cells expressing AGS3, modest, but significant increases in maximal inhibition (67±4%) and potency ($logIC_{50}$ = -8.83±0.19) for D_{2L}DR-mediated inhibition of AC1 activity by quinpirole were observed. AC2 is conditionally stimulated (requiring co-activation of AC2 by Gαs or PKC) by Gβγ subunits following the activation of Gi/o-coupled receptors (Tsu and Wong, 1996; Yoshimura and Cooper, 1993; Zimmermann and Taussig, 1996). To study acute Gβγ subunit signaling, HEK293 cells stably expressing AC2 and the D_{2L}DR were incubated with PMA to stimulate AC2, and quinpirole to activate the D_{2L}DR (subsequently releasing Gβγ subunits). Maximal activation of the D_{2L}DR by quinpirole resulted in a robust increase in cAMP accumulation through conditional activation of AC2 (240±18% of PMA response) with a $logEC_{50}$ of -7.05±0.06 (Figure 2.3B). AGS3 co-expression did not affect the maximal activation of AC2 (250±20% of PMA response), but a significant shift in the potency ($logEC_{50}$ = -7.45±0.06) of quinpirole-potentiated cAMP accumulation

was observed as compared to cells not expressing AGS3. Though modest, the effects of AGS3 on D_{2L}DR-mediated regulation of AC1 or AC2 are consistent with enhanced Gβγ subunit modulation of each effector. Specifically, Gβγ subunits inhibit signaling mediated by AC1, but increase AC2 activity, and AGS3 expression augments the potency of these effects, presumably by binding Gαi subunits. The effects of AGS3 expression on acute D_{2L}DR-mediated AC1 or AC2 activity are contrary to other cell-based studies examining the effects of AGS3 expression on acute G protein signaling. For example, AGS3 did not alter α2-adrenergic receptor-inhibited AC activity in CHO cells (Sato et al., 2004). Perhaps more relevant to our D_2DR-AC2 studies is the observation that AGS3 failed to alter D_{2S}DR-stimulated GIRK channel activity, a Gβγ mediated signaling event (Webb et al., 2005). These findings suggest that AGS3 may display AC-specific or even effector-specific regulation of acute G protein signaling.

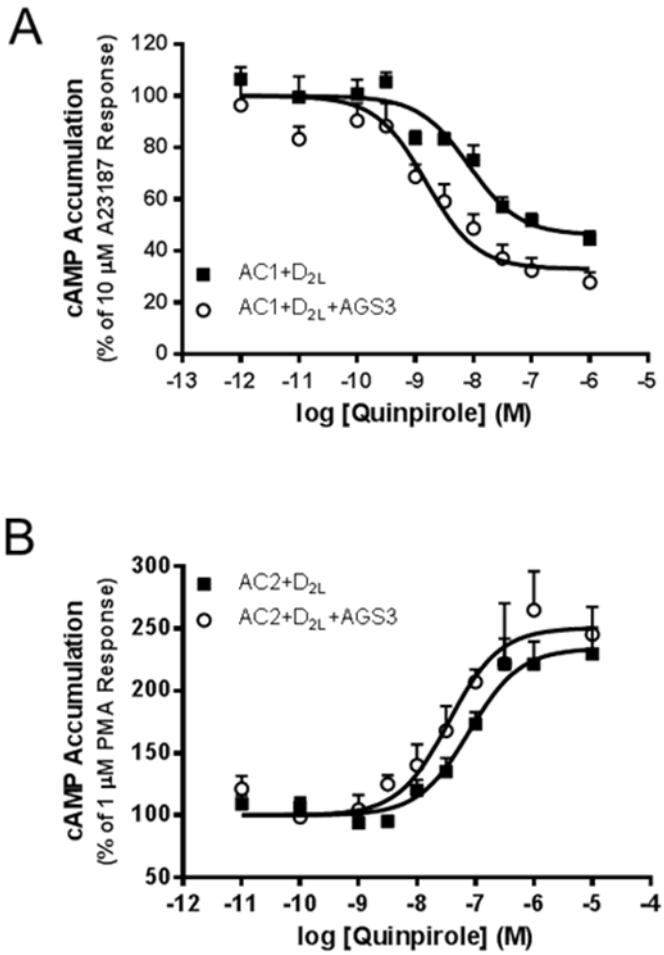

Figure 2.3 Effect of AGS3 expression on acute regulation of AC isoforms. **A.** Acute inhibition of A23187 (10 µM)-stimulated cAMP accumulation was measured following incubation with quinpirole as indicated in HEK293 cells stably expressing AC1 and the D_{2L}DR, or AC1 and the D_{2L}DR together with AGS3-Venus. **B.** Cyclic AMP accumulation was quantified in HEK293 cells stably expressing AC2 and the D_{2L}DR with or without AGS3-Venus following acute treatment with PMA (1 µM) and increasing concentrations of quinpirole as indicated. Data points represent mean ± S.E.M. of at least three independent experiments performed in duplicate.

2.3.3 Desensitization of D_{2L}DR-mediated AC1 and AC2 signaling

In addition to studying AC regulation by acute activation of the D_{2L}DR, we were also interested in studying desensitization of the D_{2L}DR. Cells stably expressing AC1 and the D_{2L}DR were treated with quinpirole for 2 h in an effort to promote functional receptor desensitization. Following washing, cells were re-exposed to quinpirole and the subsequent cAMP accumulation was measured. Persistent D_{2L}DR activation by quinpirole failed to induce significant desensitization of D_{2L}DR modulation of AC1 activity. Specifically, quinpirole treatment maximally inhibited A23187-stimulated AC1 activity by 56±9.1% with a logIC_{50} of -8.0±0.20 following persistent D_{2L}DR activation, as compared to maximum inhibition of 59±1.8% and log IC_{50} of -8.5±0.20 after vehicle pretreatment (n=2). We then examined the effects of AGS3 expression on D_{2L}DR-modulated AC1 activity following persistent D_{2L}DR stimulation. These experiments revealed that AGS3-expressing cells pretreated with quinpirole displayed a desensitization of D_{2L}DR-modulated AC1 inhibition that was manifested as a significant loss of maximal inhibition (60±6%) compared to vehicle pretreated cells (84±8%), but without a significant change in potency (logIC_{50} = -8.03±0.39 and -8.64±0.17, respectively) (Figure 2.4). The AGS3-promoted desensitization of AC1 inhibition may occur through interactions with Gαi subunits that are involved in the inhibition AC1 activity. However, it has been suggested that Gαi-AGS3 complexes can couple with, and be regulated by Gαi-coupled receptors upon agonist activation, allowing for the possibility of indirect

modulation of downstream Gβγ subunit signaling (Oner et al., 2010). Furthermore, an AGS3 consensus peptide disrupted Gβγ subunit activation of GIRK channels following repeated stimulation of D_{2S}DRs (Webb et al., 2005). Given that Gβγ subunits inhibit AC1 activity (Taussig et al., 1993b), these data present the possibility that the desensitization of AC1 inhibition is also associated with altered Gβγ subunit signaling.

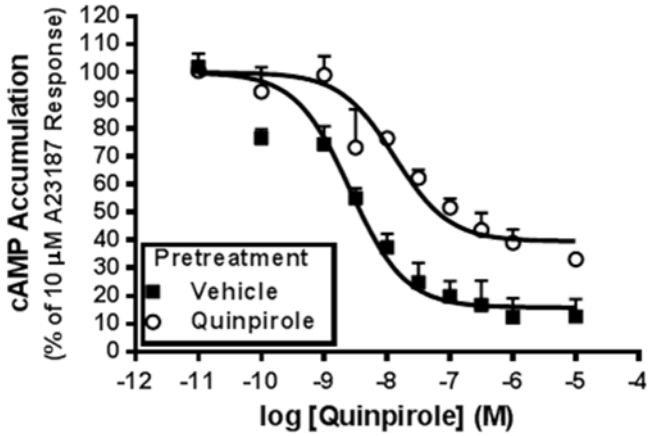

Figure 2.4 The effect of AGS3 expression on functional desensitization of D_{2L}DR-modulated AC1 activity. HEK293 cells stably expressing AC1, the D_{2L}DR, and AGS3-Venus were treated with quinpirole (1 µM) or vehicle for 2 hrs, followed by selective AC1 stimulation with A23187 (10 µM) and activation of the D_{2L}DR with quinpirole. Data represent the mean ± S.E.M. of three independent experiments.

In contrast to AC1, a complete desensitization of D_{2L}DR-mediated potentiation of AC2 activation was observed following quinpirole pretreatment in D_{2L}DR-AC2 cells. Specifically, quinpirole pretreatment resulted in 103±11% desensitization of D_{2L}DR-mediated potentiation of AC2 activity as compared to vehicle pretreatment (Figure 2.5). These observations suggest potential differences in the general mechanisms for desensitization of D_{2L}DR-modulated AC1 and AC2 activity. Such mechanisms presumably reflect the differential regulatory properties of AC1 and AC2 that involve G protein modulation (Hanoune and Defer, 2001). Subsequent experiments examined the effects of AGS3 on desensitization of D_{2L}DR-potentiated AC2 activity. Surprisingly, expression of AGS3 resulted in significantly less desensitization (46±7%) of D_{2L}DR-mediated potentiation of AC2 activity (Figure 2.5). The specificity of this blockade was probed by expressing an AGS3 mutant that does not bind Gαi subunits, AGS3-Q/A (Peterson et al., 2002). AGS3-Q/A failed to significantly alter quinpirole-induced desensitization of D_{2L}DR-AC2 signaling (78±3%), suggesting that AGS3 expression inhibits desensitization of D_{2L}DR signaling through AC2 in a manner that is dependent on the interaction of AGS3 with Gαi.

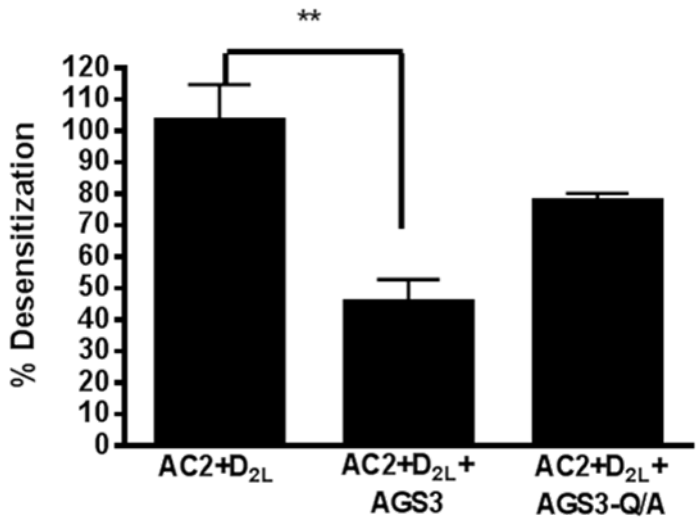

Figure 2.5 The effect of AGS3 expression on functional desensitization of D_{2L}DR-modulated AC2 activity. HEK293 cells stably expressing AC2 and the D_{2L}DR alone, or together with AGS3-Venus or AGS3-Q/A-Venus were treated with quinpirole (1 μM) or vehicle for 2 hrs, followed by stimulation with PMA (1 μM) and quinpirole (1 μM). Data represent the mean ± S.E.M. of at least three independent experiments. ** $p < 0.01$, compared with AC2+D_{2L} condition, one way analysis of variance followed by Dunnett's post hoc test.

2.3.4 Effects of AGS3 expression on heterologous sensitization of AC1 and AC2

In addition to desensitization, persistent activation of many Gi/o-coupled receptors (e.g., D2 dopamine and μ-opioid) results in enhanced subsequent AC activation. This heterologous sensitization (a.k.a. superactivation) of cAMP signaling involves both Gαi/o and Gβγ subunits (Watts and Neve, 2005), but the mechanisms of sensitization for AC1 and AC2 appear to differ (Cumbay and Watts, 2001). The functional effects of AGS3 expression on AC sensitization following persistent activation of the D_{2L}DR were examined. For these experiments, cells expressing AC1 and the D_{2L}DR were stimulated with the D_2DR agonist quinpirole for 2 h, followed by selective activation of AC1 by A23187. Quinpirole pretreatment resulted in a 235±4% enhancement of A23187-stimulated AC1 activity compared to the vehicle pretreatment (Figure 2.6A). Cells co-expressing AGS3 displayed a reduction in subsequent AC1 stimulation (178±12% of vehicle condition) following quinpirole pretreatment, suggesting that AGS3 expression attenuates AC1 sensitization by ~40%.

The effects of AGS3 expression on the regulation of AC2 following persistent D_{2L}DR activation were also explored. AC2 displayed an enhanced responsiveness (182±9% of vehicle condition) to stimulation by PMA following quinpirole pretreatment (Figure 2.6B). The D_{2L}DR-mediated sensitization of AC2 was nearly eliminated (120±3% of vehicle condition) in AGS3-expressing cells. In contrast, AGS3-Q/A did not alter D_{2L}DR-mediated sensitization of AC2 (202±10% of vehicle response), suggesting that AGS3-Gαi subunit interactions

are involved in the inhibition of AC2 sensitization. The ability of AGS3 expression to prevent heterologous sensitization of AC1 and AC2 is similar to that observed by expressing the Gβγ subunit scavenger, βARK-CT ((Koch et al., 1994; Nguyen and Watts, 2005) and see chapter 3 for AC2). These data add support to the hypothesis that AGS3 potentially alters Gβγ subunit signaling following persistent Gi-coupled receptor activation (Oner et al., 2010; Webb et al., 2005).

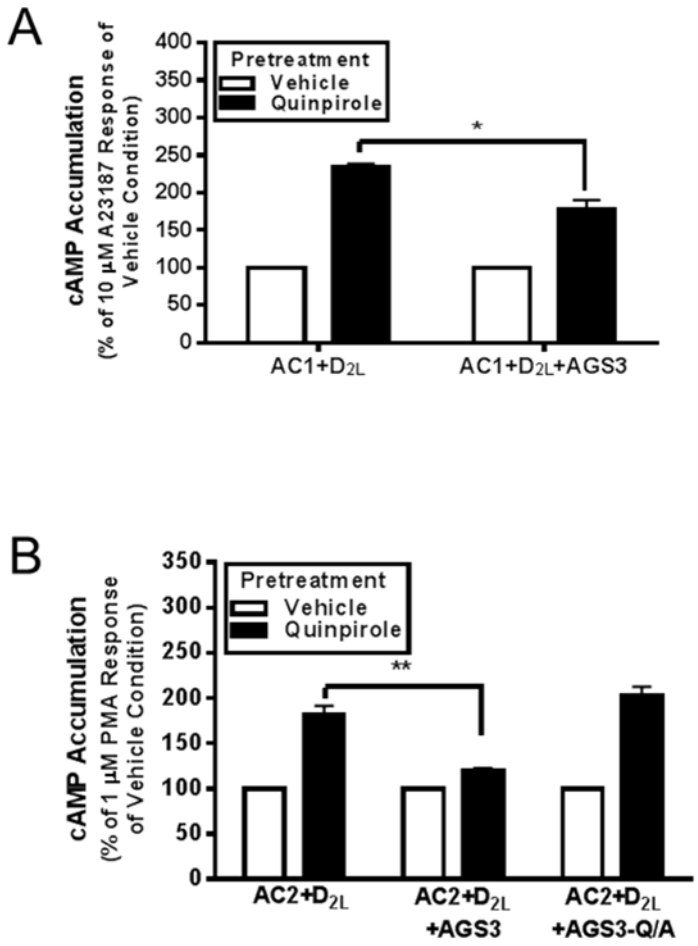

Figure 2.6 Effect of AGS3 expression on heterologous sensitization of AC isoforms. **A.** HEK293 cells stably expressing AC1 and D_{2L}DR were stimulated with vehicle or quinpirole (1 µM) for 2 hr, washed, and subsequently stimulated with A23187 (10 µM) as indicated. Data represent mean ± S.E.M. of three independent experiments. * $p < 0.05$, unpaired *t*-test. **B.** HEK293 cells stably expressing AC2 and D_{2L}DR in the absence of, or coexpressed with AGS3-Venus or AGS3-Q/A-Venus as indicated were pretreated with vehicle or quinpirole (1 µM) for 2 hr, washed, and subsequently stimulated with PMA (1 µM). Data represent mean ± S.E.M. of at least three independent experiments. ** $p < 0.01$, one way analysis of variance followed by Dunnett's post hoc test.

The inhibition of D_{2L}DR-mediated sensitization of AC1 and AC2 by AGS3 expression is consistent with a report revealing that AGS3 inhibits α2-adrenergic receptor-mediated sensitization of AC isoforms expressed in CHO cells (predominantly expressing AC6) (Sato et al., 2004; Varga et al., 1998). In contrast, evidence suggests that opioid receptor-induced up-regulation of AGS3 expression mediates sensitization of AC5 and AC7 in nucleus accumbens/striatal neurons (Fan et al., 2009). These differential effects suggest an isoform-dependence for sensitization that may reflect complex and unique isoform-specific mechanisms of AC sensitization involving Gαi, Gβγ, or a combination of the two, and subsequently altered downstream signaling. Alternatively, the isoform-specific effects on AC signaling may reflect differences in receptor types, cell types, or experimental paradigms. The differential effects of AGS3 on AC sensitization, desensitization, and acute D_{2L}DR-mediated signaling are similar to what has been observed for ligands that possess functional selectivity (or promote agonist-directed trafficking) (Kenakin, 1995). Such an observation posits that receptor- and effector-modulating signaling molecules introduce an additional level of complexity for cell signaling studies. For example, AGS3 differentially alters desensitization of D_{2L}DR-mediated modulation of AC1 and AC2, while reducing sensitization of both AC isoforms. These complex mechanisms for regulation by AGS3 *in vitro* suggest the opportunity for greater signaling diversity *in vivo*.

2.3.5 Significance of AGS3-regulated AC signaling to drugs of abuse

In vivo studies suggest that AGS3 has a "gatekeeper" role for drug-seeking behavior in response to heroin, cocaine, or ethanol. For example, knocking out AGS3 expression in specific brain regions resulted in a loss of drug-seeking behavior or cocaine-induced locomotor sensitization (Bowers et al., 2008; Bowers et al., 2004; Yao et al., 2005). Interestingly, AGS3 expression is up-regulated in the nucleus accumbens core or prefrontal cortex during withdrawal periods following prolonged ethanol or cocaine self-administration, respectively (Bowers et al., 2008; Bowers et al., 2004). In conjunction with overwhelming evidence that dopamine systems and cAMP signaling pathways are involved in drug addiction (Carlezon et al., 2005; McClung and Nestler, 2003; Nestler, 2001), our data suggest that AGS3 overexpression during periods of withdrawal may change the profile of signaling through specific AC isoforms in response to persistent Gi/o-coupled receptor activation. In addition, recent studies with AC knockout mice suggest a role for Ca^{2+}-stimulated ACs (AC1 and AC8) in the regulation of cocaine behavioral sensitization (DiRocco et al., 2009). It will be important to study the role of individual AC isoforms in specific brain regions in the context of drug-seeking behavior, where AGS3 may be exerting its effects by fine-tuning D_{2L}DR-mediated signaling through distinct AC isoforms.

Taken as a whole, our data suggest that AGS3 expression alters D_{2L}DR-mediated regulation of effector proteins in a manner that is effector-specific and dependent on the duration of Gi-coupled receptor activation. The expression of

AGS3 has modest, but potency-enhancing effects on effector modulation in response to acute Gi-coupled receptor stimulation. Expression of AGS3 promoted desensitization of prolonged D_{2L}DR-mediated signaling through AC1, but reduced desensitization of AC2 signaling. In addition, D_{2L}DR-mediated sensitization of AC1 and AC2 signaling was attenuated by AGS3 expression. It is becoming apparent that AGS3 is involved in altering G protein signaling in a complex fashion that is effector-specific

CHAPTER 3. HETEROLOGOUS SENSITIZATION OF ADENYLYL CYCLASE 2 IS DEPENDENT ON G PROTEIN BETA-GAMMA SUBUNIT SIGNALING

3.1 Introduction

AC sensitization was first reported in 1975 in the lab of Marshall Nirenberg, but an understanding of the mechanisms of AC sensitization remains elusive (Watts and Neve, 2005). However, the signaling pathways underlying AC sensitization appear to be overlapping, but distinct for each isoform (Watts and Neve, 2005). The present study focuses on sensitization of adenylyl cyclase type 2 (AC2), as AC2 is expressed in brain regions co-expressing D_2DRs and can be selectively activated in HEK293 cells (Cumbay and Watts, 2001; Visel et al., 2006; Weiner et al., 1991). AC2 is activated by Gαs and protein kinase C (PKC) phosphorylation in response to phorbol ester stimulation (e.g. PMA) and Gq-coupled receptor activation (Shen et al., 2012; Yoshimura and Cooper, 1993; Zimmermann and Taussig, 1996). Furthermore, AC2 is also conditionally activated by Gβγ subunits in response to Gi/o-coupled receptor signaling (Federman et al., 1992; Taussig et al., 1993b). In response to persistent D_{2L}DR activation, AC2 is readily sensitized, but appears to have a unique mechanism of sensitization. Specifically, AC2 enzymatic activity is enhanced in response to

PKC-mediated activation, but not Gαs- or forskolin-mediated stimulation (Cumbay and Watts, 2001). The mechanism of AC2 sensitization, however, has been largely unexplored. An understanding of the molecular adaptations that underlie D2 dopamine receptor-mediated sensitization of AC2 signaling would provide insight into the diverse modulation of cAMP signaling through AC isoforms and contribute to our understanding of the pathophysiology and potential therapeutic targeting of neurological disorders including drug abuse.

Gβγ subunit signaling is necessary for sensitization of AC isoforms including AC1 (Nguyen and Watts, 2005), AC5 (AvidorReiss et al., 1996), and AC6 (Thomas and Hoffman, 1996), but has been unexplored for D_{2L}DR-mediated sensitization of AC2. We have demonstrated that D_{2L}DR-mediated sensitization of AC2 is inhibited by expression of AGS3 and it is hypothesized that AGS3 expression may lead to disrupted Gβγ signaling in response to persistent Gi/o-coupled receptor activation (Chapter 2). Thus, it is possible that sensitization of AC2 in response to long-term D_{2L}DR activation proceeds via Gβγ subunit signaling. We directly examined the role of Gβγ subunits in the development of D_{2L}DR-mediated sensitization of AC2 with a Gβγ subunit sequestering protein, two small molecule Gβγ signaling inhibitors, and a cell permeable peptide inhibitor of Gβγ. We also pharmacologically investigated the roles of several downstream Gβγ subunit effectors in the development of AC2 sensitization.

3.2 Materials and Methods

3.2.1 Materials

[3H]-cAMP (33 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Spiperone, (±)-quinpirole, 3-isobutyl-1-methylxanthine (IBMX), G418, Dulbecco's modified Eagle's medium (DMEM), and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was purchased from Tocris Bioscience (Ellisville, MO). Lipofectamine 2000, antibiotic-antimycotic solution, and Zeocin were purchased from Invitrogen (Carlsbad, CA). FetalClone I (FCI) serum, bovine calf serum (BCS), Hank's balanced salt solution (HBSS), and Earle's balanced salt solution (EBSS) were purchased from Hyclone (Logan, UT).

3.2.2 Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 5% FCI, 5% BCS, and 1% antibiotic-antimycotic solution and maintained in a humidified incubator at 37°C and 5% CO2. For transient transfections, HEK293 cells were seeded into 96-well plates and allowed to grow to 70% confluency. Transient transfection was carried out for 48 h with Lipofectamine 2000 according to the manufacturer's protocol. The generation, characterization, and maintenance of HEK293 cells stably expressing AC2 and the D_{2L}DR was described in chapter 2.

3.2.3 Cyclic AMP accumulation assay

Cells were grown to confluency in either 48-well or 96-well plates and cAMP assays were performed on ice in assay buffer (EBSS containing 15 mM Na^+-HEPES, 2% BCS, and 0.02% ascorbic acid). For acute cAMP accumulation experiments, cells were stimulated at 37°C for 15 minutes in the presence of 500 μM IBMX. The stimulation buffer was decanted and cells were lysed with ice-cold 3% trichloroacetic acid. The plate was stored at 4°C for at least 1 h before cAMP quantification. For persistent receptor activation experiments, cells were grown to confluency in 48-well plates and pretreated in assay buffer with either vehicle or 1 μM (±)-quinpirole for 2 h at 37°C and 5% CO2. Cells were washed with assay buffer three times (3 minutes each), and subsequent stimulation was achieved by selective activation of AC2 (1 μM PMA) in the presence of 500 μM IBMX and 1 μM spiperone to block residual agonist binding from the pretreatment.

Heterologous sensitization experiments with pharmacological inhibitors were carried out in HEK293 cells stably expressing AC2 and the D_{2L}DR. Cells were seeded into 96-well plates and grown to confluency. Cell growth media was decanted and cells were pretreated with pharmacological inhibitors in Hank's balanced salt solution (HBSS), 20 mM HEPES, and 0.1% fatty acid free bovine serum albumin for 15 min at 37°C and 5% CO2. Cells with pharmacological inhibitors were then pretreated for an additional 2 h with either vehicle or 100 nM (±)-quinpirole. Subsequent AC2 activation was carried out by stimulating the cells with 1 μM PMA in the presence of 500 μM IBMX and 1 μM spiperone at

37°C and 5% CO2 for 15 min. The cells were lysed with 3% trichloroacetic acid and stored at 4°C for at least 1 h before cAMP quantification.

3.2.4 Cyclic AMP quantification

Cyclic AMP was quantified using a competitive binding assay (Przybyla and Watts, 2010). Duplicate samples of lysate from the cAMP accumulation assay were added to reaction tubes, followed sequentially by [3H]-cAMP (~1 nM final concentration), and cAMP-binding protein (~100 µg of crude bovine adrenal extract) in 500 µL of cAMP binding buffer (100 mM Tris-HCl, 100 mM NaCl, 3 mM EDTA, pH 7.4). The assay was carried out at 4°C for 2 h and harvested by filtration through Millipore FB 96-well filter plates, and radioactivity was quantified on a TopCount NXT scintillation counter (Perkin Elmer). Cyclic AMP concentrations were estimated from a standard curve ranging from 300 pmol to 3 nmol cAMP.

3.3 Results

Our initial sensitization experiments employed HEK293 cells that were transiently transfected with AC2 and the D_{2L}DR. Consistent with significant AC2 expression and function, HEK293 cells co-expressing AC2 and the D_{2L}DR displayed a robust increase in cAMP accumulation in response to PMA activation of PKC (2.75±0.48 pmol/well, n = 3) as compared to cells transfected with empty

vector (0.60±0.06 pmol/well, n = 3). To observe D_{2L}DR-mediated sensitization of AC2, cells were pretreated for 2 h with the D_{2L}DR agonist quinpirole, and subsequently stimulated with PMA to activate AC2. The quinpirole-pretreated cells co-expressing AC2 and the D_{2L}DR displayed PMA-stimulated cAMP accumulation that was 251±34% of the vehicle pretreatment condition, whereas the empty vector transfected cells provided a response that was similar to that of vehicle-treated cells (Figure 3.1). These observations are consistent with our previous studies using cells stably expressing AC2, where persistent D_{2L}DR activation resulted in a sensitized response to AC2 activation via PKC as measured by the enhancement of cAMP accumulation over that of the vehicle pretreatment condition (Chapter 2 and (Cumbay and Watts, 2001)). Studies have suggested an important role for Gi/o protein subunits in the development of AC sensitization (Watts and Neve, 2005). The regulatory properties of AC2 offer a unique system to dissociate the functional roles of Gαi/o and Gβγ subunits in AC sensitization. Specifically, AC2 is thought to be insensitive to functional regulation by Gαi subunits (Tang and Gilman, 1991; Taussig et al., 1994), thereby allowing the specific observation of Gβγ subunit-modulated AC2 activity. To directly study the role of Gβγ subunits in AC2 sensitization, the membrane-localized Gβγ subunit-sequestering protein βARKct-CD8 was co-expressed with AC2 and the D_{2L}DR in HEK293 cells. Expression of βARKct-CD8 resulted in a blockade of D_{2L}DR-mediated sensitization of AC2 (124±13% of vehicle response), suggesting a role for Gβγ subunits in sensitization of AC2 (Figure 3.1).

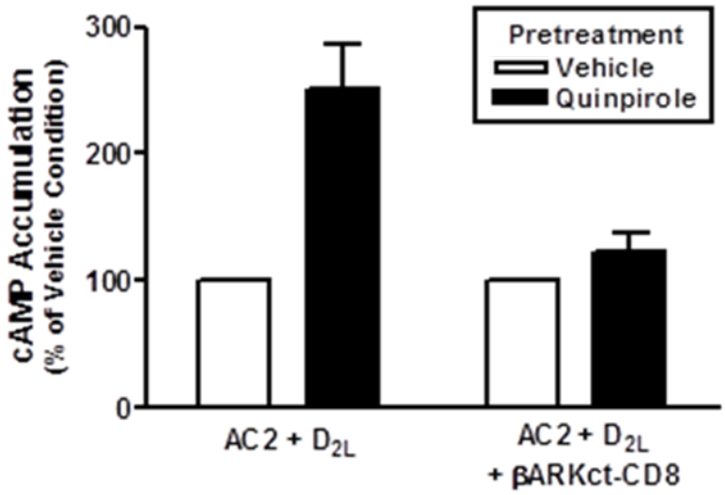

Figure 3.1 Effect of the Gβγ subunit sequestering protein on heterologous sensitization of AC2. HEK293 cells were transiently transfected with AC2, the D_{2L}DR, and either empty vector or βARKct-CD8. Cells were pretreated with quinpirole or vehicle for 2 h and subsequently stimulated with 1 μM PMA. Data are expressed as a percentage of the vehicle condition for each transfection and are the mean ± S.E.M. of three independent experiments. The raw cAMP values for each condition are as follows: AC2+D_{2L} vehicle pretreatment condition = 2.75 ± 0.48 pmol/well; AC2 + D_{2L} quinpirole pretreatment condition = 6.97 ± 1.55 pmol/well; AC2 + D_{2L} + βARKct-CD8 vehicle pretreatment condition = 2.73 ± 0.78 pmol/well; AC2 + D_{2L} + βARKct-CD8 quinpirole pretreatment condition = 3.31 ± 0.89 pmol/well.

The observation that βARKct-CD8 prevented heterologous sensitization suggests that the role of Gβγ subunits in AC2 sensitization could be either direct or indirect. In an effort to explore the direct pathway, we tested two small molecule Gβγ signaling inhibitors for their ability to modulate sensitization of AC2. HEK293 cells stably expressing AC2 and the D_{2L}DR (HEK-AC2/D_{2L} cells) were pretreated with increasing concentrations of the agonist quinpirole, followed by subsequent AC2 activation by PMA treatment. As expected, quinpirole pretreatment resulted in a concentration-dependent enhanced responsiveness of AC2 to activation by PMA (Figure 3.2A). Initial studies with the small molecule inhibitors, M119 and gallein, revealed that the quinpirole-induced enhanced response to PMA was not altered by the small molecule Gβγ signaling inhibitors. The lack of efficacy of M119 and gallein may represent the reported specificity for inhibition of specific Gβγ-effector interfaces (Bonacci et al., 2006). Thus, we examined the ability of M119 and gallein to block conditional acute activation of AC2 by Gβγ subunits (Federman et al., 1992; Taussig et al., 1993b). For these studies HEK-AC2/D_{2L} cells were treated with PMA (to activate AC2) in the presence of quinpirole to activate the D_{2L}DR (allowing for activation of Gαi and release of Gβγ subunits) for Gβγ-dependent potentiation of AC2 activity. The results of these studies revealed that the Gβγ signaling inhibitors, M119 and gallein, had no effect on either PMA-stimulated or Gβγ-dependent potentiation of AC2 (Figure 3.2B).

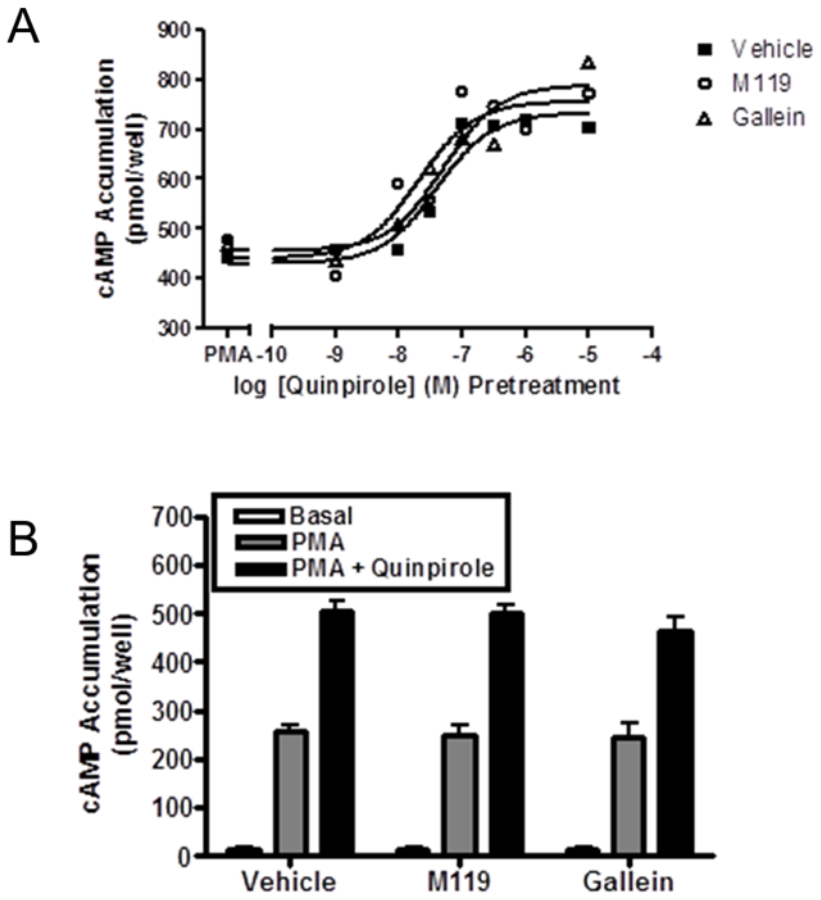

Figure 3.2 The effect of Gβγ signaling inhibitors on D2LDR-mediated AC2 signaling. **A.** The effect of M119 and gallein (10 μM) on quinpirole-induced sensitization was measured in HEK-AC2/D_{2L} cells. Cells were pretreated with increasing concentrations of quinpirole for 2 h in the presence of vehicle, M119, or gallein and subsequently stimulated with 1 μM PMA. Data are representative of two independent experiments. **B.** The effect of M119 and gallein (10 μM) on acute quinpirole-induced potentiation of PMA-stimulated AC2 activity was measured in HEK-AC2/D_{2L} cells. Data are the mean ± S.E.M. of three independent experiments.

In an effort to rapidly assess the activity of several small molecules for modulation of D_{2L}DR-induced sensitization of AC2, we developed a 96 well sensitization assay (with reduced wash and decant steps) for increased throughput (Conley et al., *in press*). The higher throughput sensitization assay provided the ability to efficiently investigate multiple pharmacological modulators simultaneously. The observation that βARKct-CD8 expression inhibits D_{2L}DR-induced sensitization of AC2 and lack of effect by M119 and gallein suggest the hypothesis that AC2 sensitization is mediated by Gβγ subunits, in an indirect fashion, by a downstream effector of Gβγ subunits. Furthermore, previous sensitization studies with other adenylyl cyclases have provided evidence that a number of kinases may also be involved in the development and expression of sensitization of adenylyl cyclase (Chakrabarti et al., 1998; Johnston et al., 2002). Thus, several ligands including a peptide Gβγ signaling inhibitor, small molecule inhibitors of Gβγ effectors, and several additional kinase inhibitors were tested for the ability to inhibit quinpirole-induced sensitization of AC2 in HEK-AC2/D_{2L} cells. Specifically, HEK-AC2/D_{2L} cells were pretreated with inhibitors in the presence of either vehicle or quinpirole, and subsequently stimulated with PMA to promote PKC activation of AC2. The results of these studies are depicted as a percentage of sensitization (i.e., quinpirole induced sensitization = 100%). Consistent with the effects of βARKct-CD8, the cell-permeable Gβγ subunit sequestering peptide, QEHA-TAT significantly inhibited sensitization in the HEK-AC2/D_{2L} cells, whereas the small molecule Gβγ subunit inhibitor, gallein, had no effect (negative control) (Table 3.1). The next set of compounds included

reported pharmacological kinase inhibitors that target Gβγ effectors that include c-JNK, Raf-1, PI3K, or MEK. Much to our disappointment, there was no significant difference observed in the D_{2L}DR-mediated sensitization of AC2 in the presence of the Gβγ-modulated kinase inhibitors (Table 3.1). Additional kinases, including those that have been identified as being involved in sensitization of AC isoforms (i.e., PKC and PKA) were also examined in the same manner (Chakrabarti et al., 1998; Johnston et al., 2002). As expected, the PKC inhibitor bisindolylmaleimide I (BisI) blocked sensitization of AC2 (3.0±1.7% sensitization). Inhibitors of PKA (H89) and PI3K/PI4K (phenylarsine oxide, PAO) significantly reduced the level of D_{2L}DR-mediated sensitization of AC2 (29±12% and 29±5% sensitization, respectively), suggesting roles for these kinases in sensitization of AC2.

Table 3.1 Effects of pharmacological inhibitors on D_{2L}DR-mediated sensitization of AC2. Data are expressed as a percent sensitization with the quinpirole-induced sensitization = 100%. Data are the mean ± S.E.M. of five independent experiments.

Compound	**Target**	**Mean±S.E.M.**
Control		100
10 μM Gallein	Gβγ	110±24
30 μM QEHA-TAT	Gβγ	62±8.2*
10 μM SP600125	c-JNK	130±13
30 μM GW5074	Raf-1	99±12
300 nM Wortmannin	PI3K	95±10
30 μM PD98059	MEK	90±6.2
10 μM H89	PKA	29±12 ***
10 μM PAO	PI3K/PI4K	29±5.5 ***
1 μM BisI	PKC	3.0±1.7 ***

3.4 Discussion

G protein subunits are known to be important components of the signaling pathways that contribute to the development of heterologous sensitization of multiple isoforms of adenylyl cyclase (Watts and Neve, 2005). Studies with pertussis toxin and pertussis-toxin insensitve Gαi/o subunits have indicated essential roles for Gαi/o subunit activation in AC sensitization (Watts and Neve, 1996; Watts et al., 1998), but mounting evidence also suggests that Gβγ subunits play an important part in the development of AC sensitization (AvidorReiss et al., 1996; Nguyen and Watts, 2005; Rhee et al., 2000; Rubenzik et al., 2001). As most ACs are differentially regulated by Gαi/o and Gβγ subunits, the precise roles of these subunits in sensitization of cAMP signaling have been difficult to examine (Hanoune and Defer, 2001; Patel et al., 2001). Nevertheless, AC2 shows only conditional activation by Gβγ subunits and is not directly regulated by Gαi/o subunits (Tang and Gilman, 1991; Taussig et al., 1994). These unique regulatory properties of AC2 allow for the selective study of Gβγ subunit signaling in sensitization of PKC-stimulated AC2 activity. The present data suggest that similar to other AC isoforms, Gβγ subunits are necessary for the sensitization of AC2. Specifically, the membrane-localized Gβγ subunit sequestering protein βARKct-CD8 attenuated D_{2L}DR-mediated sensitization of AC2. Sensitization of AC2 was also reduced using a cell-permeable peptide inhibitor of Gβγ signaling, QEHA-TAT. The peptide sequence corresponds to a Gβγ subunit-binding sequence derived from the C2 domain of AC2 and is known inhibit Gβγ subunit

signaling (Chen et al., 1995a). Our data are in agreement with our previous study, where AGS3 blocked D_{2L}DR-mediated sensitization of AC2 (Chapter 2). AGS3 is hypothesized to disrupt Gβγ subunit signaling in response to long-term or repeated Gi-coupled receptor signaling. These findings, along with the unique properties of AC2 suggest that D_{2L}DR activation leads to a Gβγ-dependent event that selectively enhances PKC-mediated activation of AC2.

Two small molecule Gβγ subunit signaling inhibitors, M119 and gallein were also studied for their effects on D_{2L}DR-mediated sensitization of AC2. Surprisingly, neither small molecule (up to 10 μM) altered sensitization of AC2 or D_{2L}DR-potentiated AC2 activity (a Gβγ subunit-mediated effect). The lack of activity of these compounds was discouraging, but may be explained by the effector specificity profiles displayed by the small molecule inhibitors (Bonacci et al., 2006). The small molecule inhibitors selectively bind to a common region of Gβγ, known as the “Gβγ hot spot”, that interacts with Gα subunits and many Gβγ effector proteins (Ford et al., 1998; Hamm, 1998; Li et al., 1998; Lin and Smrcka, 2011). It is thought that different residues or regions within the hot spot are important for binding different effectors (Ford et al., 1998; Li et al., 1998). It is possible that M119 and gallein do not interact with the region of Gβγ that mediates acute potentiation of AC2 signaling.

Several studies have contributed evidence that suggests AC2 has multiple Gβγ subunit binding sites (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006; Weitmann et al., 2001). Specifically, at least five distinct sites have been identified within C1a, C1b, and C2 regions of AC2 for their interaction with Gβγ

subunits (see Figure 1.6) (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006; Weitmann et al., 2001). It is therefore possible that persistent activation of the D_{2L}DR releases Gβγ subunits that directly interact with AC2, allowing a conformation that provides an enhanced response to activation by PKC. The identification of multiple Gβγ binding sites makes it tempting to speculate that the Gβγ sites for sensitization of AC2 may be unique from those involved with conditional activation.

Gβγ subunits regulate a multitude of downstream effectors (Khan et al., 2013; Smrcka, 2008). Therefore, it is possible that Gβγ subunit signaling indirectly leads to sensitization of AC2 via modulation of downstream Gβγ effectors. We addressed this possibility by examining the ability of pharmacological inhibitors of Gβγ subunit effectors to modulate D_{2L}DR-mediated sensitization of AC2 in HEK-AC2/D_{2L} cells. The concentrations of pharmacological inhibitors tested had no significant effect on D_{2L}DR-mediated AC2 sensitization. However, upon treatment of HEK-AC2/D_{2L} cells with H89 or PAO, significant inhibition of AC2 sensitization was observed, suggesting roles for PKA and PI4K. Interestingly, subsequent studies with PAO revealed a biphasic potentiation of acute AC2 stimulation in response to PMA treatment (data not shown) and no effect of additional PI4K inhibitors (LY294002 and Pik93, John Paul Spence and Val J. Watts, *unpublished results*). These results suggest PAO modulation of D_{2L}DR-mediated sensitization of AC2 is complex and perhaps independent of its effects on PI4K. In summary, our data suggest that PKA and

PKC mediate sensitization of AC2 in response to persistent D_{2L}DR activation by quinpirole.

The present study leveraged the unique regulatory properties of AC2 (i.e., lack of direct modulation by Gαi/o subunits and selective sensitization to PKC activation) to demonstrate that Gβγ subunits are required for AC sensitization. However, the mechanism underlying the enhancement of PKC-stimulated AC2 remains unknown. Ongoing efforts to identify signaling components that mediate AC sensitization include the development of higher throughput cell-based cAMP detection methodology to facilitate unbiased approaches such as siRNA library screening and small molecule screening (e.g., known kinase inhibitor sets). These approaches are expected to expand the repertoire of signaling molecules implicated in sensitization.

CHAPTER 4. DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING PARADIGM FOR THE DISCOVERY OF SMALL MOLECULE MODULATORS OF ADENYLYL CYCLASE: IDENTIFICATION OF AN ADENYLYL CYCLASE 2 INHIBITOR

4.1 Introduction

Cyclic AMP (cAMP) is a crucial component of signal transduction cascades that modulates diverse fundamental biological processes (Hanoune and Defer, 2001). The cellular levels of cAMP are dynamically modulated by two families of enzymes. Specifically, adenylyl cyclases (ACs) synthesize cAMP from ATP (Hanoune and Defer, 2001) and phosphodiesterase enzymes degrade cAMP (Bender and Beavo, 2006). The specificity of cAMP signaling is influenced by the interplay of signaling molecules that are expressed within a given cell. For example, nine membrane-bound mammalian AC isoforms have been identified that have unique profiles of regulation by G protein subunits, protein kinases, and Ca^{2+} (Hanoune and Defer, 2001; Patel et al., 2001). Specifically, the AC isoforms are commonly activated by the stimulatory G protein (Gs), but are categorized into four subgroups based on their sequence similarities and regulatory properties (Hanoune and Defer, 2001; Patel et al., 2001). The group I ACs (AC1, AC3, and AC8) are stimulated by Ca^{2+}/calmodulin. In contrast, group

II ACs (AC2, AC4, and AC7) are insensitive to Ca^{2+}, but conditionally activated by G protein Gβγ subunits. The group III ACs (AC5 and AC6) are inhibited by free Ca^{2+}, Gαi/o subunits, and phosphorylation by protein kinase A. AC9, the lone member of group IV is distinguished by its relative insensitivity to stimulation by forskolin.

Insight from knockout and transgenic mouse studies suggest that individual AC isoforms contribute to important physiological processes and diseases (Sadana and Dessauer, 2009), suggesting that selective AC modulators have therapeutic utility for the treatment of conditions involving cardiac function, aging, and pain. As such, the AC5/AC6 inhibitors PMC-6 and AraAde have shown efficacy in preventing cardiomyocyte apoptosis (Iwatsubo et al., 2004) and a mouse model of heart failure, respectively (Iwatsubo et al., 2012). Also, a small molecule AC1 inhibitor, NB001, has been reported to have analgesic effects in animal models of neuropathic and inflammatory pain (Vadakkan et al., 2006; Wang et al., 2011). However, a dearth of isoform-selective small molecule AC modulators has limited the study of AC isoforms as therapeutic targets (Pierre et al., 2009; Seifert et al., 2012). For example, AC2 is potentially involved in skeletal muscle physiology, lung diseases, neuroendocrine tumors (NETs), and colorectal cancer (Berdeaux and Stewart, 2012; Drozdov et al., 2011; Duerr et al., 2008; Yu et al., 2011). Yet, the pharmacological study of AC2 is difficult because most small molecule AC inhibitors preferentially inhibit other AC isoforms (Pierre et al., 2009; Seifert et al., 2012). BODIPY-forskolin appears to be the most potent AC2 inhibitor, but its use as a chemical probe for

AC2 activity is hindered because it also partially activates AC1 and AC5 (Erdorf et al., 2011; Pinto et al., 2008). Given the shortcomings of small molecule AC modulators and the absence of published reports of AC2 knockout animals, the identification of selective AC2 modulators is expected to provide useful chemical probes to facilitate the study of AC2.

The present report describes the development and execution of a cell-based screening approach for the discovery of novel small molecule inhibitors of AC2. We screened the NIH clinical collections I and II (727 compounds) for small molecules that inhibit cAMP accumulation in response to selective activation of AC2. Compounds identified as active were examined in a series of confirmation assays to validate direct AC2 inhibition and define their AC isoform-selectivity profiles. Our studies have resulted in the identification of SKF-83566 as a selective AC2 inhibitor that is expected to be a promising tool to investigate the physiological roles of AC2.

4.2 Materials and methods

4.2.1 Materials

The NIH clinical collections I and II were purchased from Evotec, Inc (South San Francisco, CA). Oxymetholone, tranilast, amlexanox, duloxetine, and indatraline were purchased from Sequoia Research Products (Pangbourne, United Kingdom). [^{3}H]-cAMP was purchased from PerkinElmer Life and

Analytical Sciences (Boston, MA). A23187, 3-isobutyl-1-methylxanthine (IBMX), loratadine, prochlorperazine, maprotiline, thioridazine, G418, Dulbecco's modified Eagle's medium (DMEM), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA), forskolin, MDL-12,330A HCl, prostaglandin E_2 (PGE_2), SQ22,536, and (±)-SKF-83566 HBr were purchased from Tocris Bioscience (Ellisville, MO). 2'5'-dideoxyadenosine was purchased from Santa Cruz Biotechnology (Dallas, TX). Lipofectamine 2000, opti-MEM, and antibiotic-antimycotic 100x solution were purchased from Life technologies (Grand Island, NY). FetalClone I serum, bovine calf serum, HEPES, and Hank's balanced salt solution (HBSS) were purchased from Hyclone (Logan, UT). BisindoloylmaleimideI (BisI) was purchased from Calbiochem (La Jolla, CA). The HTRF cAMP and Cellul'ERK kits were purchased from Cisbio Bioassays (Bedford, MA).

4.2.2 Stable cell line generation and cell culture conditions

HEK293 cells were cultured in DMEM supplemented with 5% bovine calf serum, 5% fetal clone I, and 1% antibiotic-antimycotic 100x solution and maintained in a humidified incubator at 37°C and 5% CO_2. For generation of a clonal stable cell line, HEK293 cells were transfected with pcDNA3.1(+) encoding human AC1, AC2, or AC5 using Lipofectamine 2000 according to the manufacturer's protocol. Stable clones were selected by growth in media containing 600 µg/ml (AC2) or 800 µg/ml (AC1 and AC5) G418. Stable

expression of AC isoforms was confirmed functionally by measuring cAMP accumulation to selective pharmacological activation conditions. For example, AC1 was stimulated with 3 µM A23187, AC2 was stimulated with the phorbol ester, PMA, and AC5 was activated by 300 nM forskolin.

The C2C12 mouse skeletal muscle cell line was purchased from the American Type Culture Collection. C2C12 myoblasts were maintained at a low confluency in DMEM media containing 10% fetal bovine serum. Myoblasts (passages 3-17) were plated in 96-well format at 5x104 cells per well. Differentiation into myotubes was induced once the cells reached 90% confluency by switching to medium supplemented with 2% horse serum. The growth medium was changed every 24 hours. Myotubes were allowed to mature for 5 days before being experiments were completed.

4.2.3 Cisbio HTRF cAMP assay

The cellular cAMP levels were measured using either the Cisbio HTRF cAMP dynamic 2 assay kit or a dynamic 2/HiRange hybrid kit (consisting of cAMP-d2 from the dynamic 2 kit and the anti-cAMP cryptate conjugate from the HiRange kit). The cAMP assays were performed on cryopreserved cells that were rapidly thawed at 37°C and resuspended in cell suspension buffer (HBSS, 20 mM HEPES, 0.1% fatty acid free BSA or opti-MEM for HEK-hAC1 cells). Cells were centrifuged at 500×g and the supernatant was aspirated. Cells were washed by resuspending in cell suspension buffer and centrifuged at 500×g. The

supernatant was aspirated and cells were seeded into a 384-well plate and allowed to incubate at 37°C and 5% CO_2 for 2.5 h. Cells were then treated as indicated with ligands diluted in stimulation buffer (HBSS, 20 mM HEPES, 500 µM IBMX or opti-MEM, 500 µM IBMX for HEK-hAC1 cells) and incubated for 1 h at room temperature. The stimulation was terminated by sequential addition of 10 µl/well of cAMP-d2 and 10 µl/well of anti-cAMP cryptate conjugate, each diluted (1:39) in lysis buffer. The experiments that used the dynamic 2 kit for cAMP detection were performed without IBMX in the stimulation buffer (to accommodate the sensitivity for cAMP detection), but with IBMX in the lysis buffer (to prevent phosphodiesterase-mediated degradation of cAMP in the lysate). Following a 1 h incubation at room temperature, the time-resolved fluorescence energy transfer (TR-FRET) was measured with a lag time of 100 µs and integration time of 300 µs using a Synergy4 (BioTek) fluorescence plate reader (excitation filter: 330/80 nm and emission filters: 620/10 nm and 665/8 nm). The resulting cAMP concentrations were calculated in GraphPad Prism by applying the 620/665 nm fluorescence ratio values to a standard curve of known cAMP concentrations.

4.2.4 Screening conditions

Cryopreserved HEK-hAC2 cells were seeded into a 384-well plate at 15 µl/well using a MultiFlo (Biotek) bulk reagent dispenser. Following a 2.5 h incubation at 37°C and 5% CO_2, the cell plates were allowed to equilibrate to

room temperature on the bench for 15 min. Test compounds (80 nl) were added to the cells with a MultiPette-mounted 384-well pin tool and allowed to incubate at room temperature for 30 min. AC2 activity was then stimulated by addition of 5 µl of PMA (50 nM final concentration) diluted in stimulation buffer with the MultiFlo reagent dispenser, followed by incubation at room temperature for 1 h. The Cisbio HTRF cAMP dynamic 2 kit was used to quantify the cellular cAMP as described above. Briefly, the cAMP-d2 (containing 500 µM IBMX final volume) and anti-cAMP cryptate conjugate working reagents were sequentially added (10 µl/well each) with a MultiFlo reagent dispenser and incubated at room temperature for 1 h. Test compounds were initially screened in singlet or duplicate and all cAMP concentrations were converted to percent inhibition of the PMA-stimulated cAMP response.

4.2.5 3H-cAMP assay

Cryopreserved HEK-hAC2 cells were thawed and prepared as described above. Cells were seeded at a density of 12,000 cells per well into a 384-well plate in cell suspension buffer and incubated for 2.5 h at 37°C and 5% CO2. Test compounds were added to the cells with a MultiPette-mounted 384-well pin tool and allowed to incubate at room temperature for 30 min. The AC stimulation was carried out at room temperature for 1 h and the reaction was stopped by the addition of cold (4°C) TCA to provide a final TCA concentration of 3%. The cAMP in the lysate was then quantified using a 3H-cAMP competition assay as

previously described (Przybyla and Watts, 2010). C2C12 cell cAMP experiments were performed similarly, with the exceptions that the assay was performed in 96-well format on continuously propagated cells before differentiation into myotubes and compounds were delivered by multi-channel pipette for both pretreatment and stimulation steps.

4.2.6 ERK1/2 phosphorylation assay

HEK-hAC2 cells were seeded into 96-well plates at a density of 25,000 cells per well in opti-MEM and incubated overnight at 37°C and 5% CO_2. Drug treatment was carried out in opti-MEM as follows. Cells were pretreated with test compound (30 µM) at 37°C for 10 min and ERK1/2 phosphorylation was stimulated by the addition of PMA (50 nM) and incubation for 10 min at 37°C. The resulting ERK1/2 phosphorylation was measured using the Cisbio htrf Cellul'ERK assay according to the manufacturer's protocol (two-plate protocol). Briefly, the stimulation buffer was decanted and supplemented lysis buffer was added, followed by shaking at 500 rpm at room temperature for 30 min. The anti-ERK-Eu^{3+}-cryptate and anti-phospho-ERK-d2 antibodies were combined and added to a 384-well low-volume plate (PerkinElmer, white, Proxiplate 384 Plus). Lysate from the stimulation or positive/negative control lysate was then added on top of the HTRF reagents and incubated for 2 h at room temperature. The TR-FRET was then measured on the Synergy4 (BioTek) plate reader.

4.3 Results

4.3.1 Assay development and screening of the NIH clinical collections I and II

The lack of robust inhibitors of AC2 (Pavan et al., 2009; Pierre et al., 2009; Seifert et al., 2012) and the absence of published reports on $AC2^{-/-}$ mice suggest that the discovery of AC2 inhibitors will provide important research tools. Thus, one initial goal of the present study was to develop an approach to identify and validate novel inhibitors of AC2 activity in intact cells. To achieve this goal, we developed and optimized assay parameters for the measurement of intracellular cAMP in 384-well format in a semi-automated fashion, a tactic that may ultimately allow for large-scale high-throughput screening to identify novel AC2 inhibitors.

HEK293 cells are known to endogenously express multiple AC isoforms (Hellevuo et al., 1993; Ludwig and Seuwen, 2002). Therefore, to specifically study AC2 modulation, it is important to identify pharmacological stimulation conditions that selectively activate recombinant AC2 when expressed in HEK293 cells. We and others have previously reported that the protein kinase C (PKC)-activating phorbol ester, PMA, selectively stimulates cAMP accumulation in HEK293 cells stably expressing recombinant rat AC2 (Cumbay and Watts, 2001; Yoshimura and Cooper, 1993). For this study, HEK293 cells stably expressing human AC2 (hAC2) were constructed and screened for cAMP accumulation in response to PMA. It is notable that the basal level of cAMP in the HEK-hAC2 cells was higher than the HEK-wt cells (data not shown) and is likely due to

constitutive activity of AC2, a property that has been previously observed (Pieroni et al., 1995; Pinto et al., 2008). As expected, PMA treatment had no effect on cAMP in HEK-wt cells, but provided an ~8 fold enhancement of cAMP in HEK-hAC2 cells (data not shown and Figure 4.1A). These results suggest that recombinant hAC2 can be selectively activated by PMA in an HEK293 cell background.

Following the verification of PMA treatment as a strategy for selective activation of AC2, potential screening parameters were further explored by performing a more in-depth evaluation of the effects of PMA in HEK-hAC2 cells. PMA treatment provided a concentration-dependent increase in cAMP with an EC_{50} value of 16±5.0 nM (n = 3) (Figure 4.1A). We chose to use 50 nM PMA (~EC_{85} concentration) to stimulate AC2 for the study of AC2 inhibitors. As a control for the inhibition of AC2 activity, the PKC inhibitor BisI was used to inhibit the phorbol ester-mediated activation of AC2. Treatment with BisI provided full inhibition of PMA-stimulated AC2 activity with an IC_{50} of 16±1.9 nM (n = 3), suggesting that 1 μM BisI is sufficient to completely inhibit AC2 activity stimulated by 50 nM PMA (Figure 4.1A, inset).

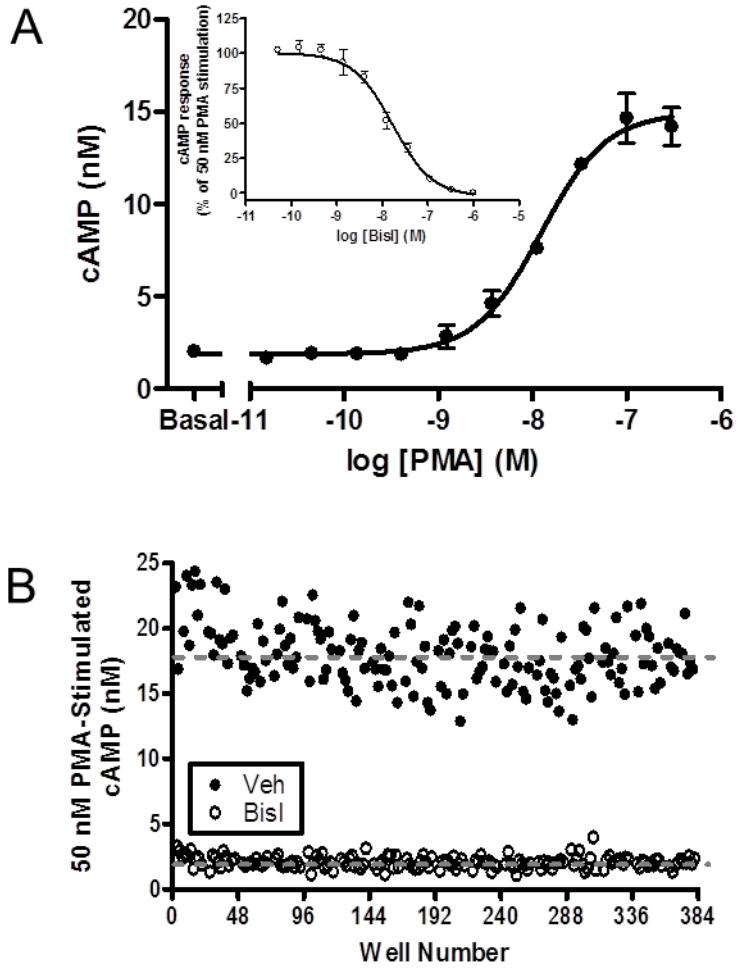

Figure 4.1 Optimization of conditions for an intact-cell assay that is capable of high-throughput screening for small molecule inhibitors of AC2. **A.** Concentration-response curve analysis of PMA for stimulation of an AC2-mediated cAMP response in HEK-hAC2 cells. Data are the mean±S.E.M. of three independent experiments. Inset: Inhibition of 50 nM PMA-stimulated AC2 activity with the PKC inhibitor BisI. Data are the mean±S.E.M. of three independent experiments**. B.** Evaluation of assay robustness by Z' analysis (Z' = ((AVGmax – 3 * SDmax/√n) – (AVGmin + 3 * SDmin/√n)) / (SDmax/√n)). Data are representative of three independent experiments.

Ultimately, our approach for the identification of AC inhibitors relies on the development of a cell-based assay that is capable of high-throughput screening of small molecule libraries. Therefore, it was important to evaluate the robustness of the HEK-hAC2 cell cAMP assay when converted to a semi-automated format that is amenable to high-throughput screening (see methods). Specifically, assay robustness was examined by performing a Z' analysis for the assay parameters developed for screening (Zhang et al., 1999). The Z' value was calculated using 50 nM PMA as the maximum stimulation control and 1 μM BisI as the minimum stimulation control. Our AC2 screening assay provided a Z' = 0.44 ± 0.02 (n = 3), suggesting that the assay is appropriate for small molecule library screening (Figure 4.1B) (NCGC Assay Guidance Manual, www.ncbi.nlm.nih.gov/books/NBK53196/).

The NIH clinical collections I and II consist of 727 total test compounds that have a history of use in human clinical trials (www.nihclinicalcollection.com). The collections contain drug-like molecules with documented biological activity and safety profile information. The modest size of the collections, paired with the reasons stated above, make the NIH clinical collections a good starting collection for early screening efforts in the search for AC2 inhibitors. The NIH clinical collections were screened for the ability of test compounds (25 μM) to inhibit PMA-stimulated AC2 activity in HEK-hAC2 cells. Of the 727 compounds screened (the full screening results are reported in the appendix, Table A.1), 10 compounds identified as active for the inhibition of PMA-stimulated cAMP

accumulation in HEK-hAC2 cells (displaying > 30% inhibition at 25 μM) were chosen for additional confirmation and validation (Table 4.1).

Table 4.1 Screening of NIH Clinical Collections I and II for inhibition of AC2 activity. The NIH Clinical Collections I and II (25 μM) were screened for inhibition of PMA-stimulated (50 nM) cAMP accumulation in HEK-hAC2 cells using the Cisbio HTRF cAMP dynamic 2 detection methodology. The data represent the average percent inhibition of the PMA-stimulated cAMP response from duplicate plates (see methods).

Compound Name	**Inhibition (%)**
SKF-83566	85
Tranilast	69
Loratadine	64
Thioridazine	58
Duloxetine	51
Amlexanox	41
Indatraline	39
Oxymetholone	37
Prochlorperazine	35
Maprotiline	33

4.3.2 Confirmation and validation of activity

Confirmation studies were carried out using freshly prepared powders that were purchased from commercial sources. The initial confirmation of active small molecules used a single concentration (30 μM) of the test compounds for the inhibition of AC2 activity. HEK-hAC2 cells were incubated with 50 nM PMA to selectively activate AC2 in the presence of test compound and the resulting cAMP accumulation was measured with the Cisbio HTRF cAMP dynamic 2 kit (identical to the assay format used for small molecule library screen). All test compounds provided inhibition of AC2 activity at 30 μM, confirming the activity observed in the initial screen (Table 4.2).

Table 4.2 Confirmation of the inhibitory activity of test compounds identified in the screen of NIH Clinical Collections I and II. Active compounds (30 µM) were tested for inhibition of PMA-stimulated cAMP in HEK-hAC2 cells using either the Cisbio HTRF cAMP dynamic 2 technology or a [3H]-cAMP competition method for detection of cAMP. Data are reported as the mean±S.E.M. of the percent inhibition of the PMA response from three independent experiments. ND, not determined.

Compound Name	**Inhibition (%) TR-FRET**	**Inhibition (%) ^{3}H-cAMP**
SKF-83566	95±1.6	94±2.8
Tranilast	76±2.9	79±5.3
Loratadine	61±0.5	71±8.1
Thioridazine	36±5.7	ND
Duloxetine	39±3.8	ND
Amlexanox	45±5.7	ND
Indatraline	60±2.0	53±5.5
Oxymetholone	58±1.9	58±2.9
Prochlorperazine	41±8.3	ND
Maprotiline	26±1.8	ND

The screening assay and initial confirmation of active compounds rely on TR-FRET for the detection of cAMP (see materials and methods). It is possible that the active compounds have inherent fluorescence that can be measured in the same wavelengths as those utilized for cAMP detection, thereby skewing the measured fluorescence and affecting the resulting estimation of the cAMP concentration (Degorce et al., 2009). Thus, we assessed the ability of the best compounds (i.e., those that provided at least 50% inhibition in the confirmation assay) to inhibit AC2 activity in a non-fluorescence-based assay. The active compounds SKF-83566, oxymetholone, tranilast, indatraline, and loratadine were tested for their ability to inhibit PMA-stimulated cAMP accumulation in a 3H-cAMP competitive binding assay. All of the compounds tested retained the ability to inhibit PMA-stimulated cAMP accumulation in HEK-hAC2 cells, indicating bona fide reduction of cAMP, rather than interference with the fluorescence detection methodology (Table 4.2). Furthermore, the extent of inhibition of PMA-stimulated AC2 activity by the test compounds in the 3H-cAMP assay was nearly identical to that observed in the TR-FRET-based cAMP detection method.

Our screening strategy utilized PMA to selectively stimulate AC2 via phosphorylation mediated by PKC (Jacobowitz and Iyengar, 1994). Thus, it is possible that the compounds identified as active may exert their cAMP-attenuating effects through inhibition of PKC rather than directly inhibiting AC2. PMA-mediated PKC activation is known to stimulate ERK1/2 phosphorylation in HEK293 cells (DellaRocca et al., 1997), allowing for a simple counter-screen to eliminate false positives (Figure 4.2A). Specifically, we measured ERK1/2

phosphorylation in response to PKC activation in HEK-hAC2 cells in the absence and presence of the active compounds (Figure 4.2B). As expected, PMA treatment resulted in a significant enhancement of ERK1/2 phosphorylation (5.7±0.2 fold over basal) that was inhibited completely by the PKC inhibitor, BisI. In contrast, SKF-83566, oxymetholone, tranilast, and loratadine did not significantly alter the PMA-mediated ERK1/2 phosphorylation in HEK-hAC2 cells, suggesting that these compounds do not inhibit PKC. Indatraline, however, inhibited the PMA-mediated ERK1/2 phosphorylation by ~80%.

The lack of inhibition of PKC-dependent ERK1/2 phosphorylation by SKF-83566, oxymetholone, tranilast, and loratadine is consistent with a direct inhibition of AC2 activity by these drugs. To further test this supposition, we examined the ability of SKF-83566, oxymetholone, tranilast, and loratadine to inhibit AC2 activity stimulated via other mechanisms. AC2 is also stimulated in a PKC-independent manner by Gαs in response to activation of Gs-coupled receptors and directly via the small molecule AC activator, forskolin. Prostaglandin E_2 (PGE_2) is known to bind and activate the Gs-coupled EP2/4 prostanoid receptors that are endogenously expressed in HEK293 cells (Bogard et al., 2012; Willoughby et al., 2007). As expected, PGE_2 treatment resulted in a concentration-dependent increase in cAMP accumulation in HEK-hAC2 cells (EC_{50}: 160±78 nM, n = 3). To examine the effects of inhibitors on Gαs-stimulated AC2 activity, the identified active compounds were then tested for the inhibition of 300 nM PGE_2-stimulated cAMP in HEK-hAC2 cells (Figure 4.2C). Oxymetholone, SKF-83566, tranilast, and loratadine significantly inhibited PGE_2-stimulated

cAMP in HEK-hAC2 cells. Next, they were similarly tested for their ability to inhibit the cAMP generated in response to direct AC stimulation by forskolin (Figure 4.2D). Oxymetholone, SKF-83566, tranilast, and loratadine significantly inhibited forskolin-stimulated cAMP accumulation in HEK-hAC2 cells. These data are in agreement with the effects of the test compounds on PKC-mediated ERK1/2 phosphorylation. Specifically, indatraline and BisI inhibited PKC-mediated ERK1/2 phosphorylation, but not Gαs- or forskolin-stimulated cAMP in HEK-hAC2 cells. Taken together, these observations suggest that inhibition of PMA-stimulated cAMP by indatraline in HEK-hAC2 cells is due to inhibition of PKC. More importantly, our data indicate that SKF-83566, oxymetholone, tranilast, and loratadine inhibit multiple modes of AC2 stimulation (i.e., PKC-, Gαs-, and forskolin-mediated), but do not inhibit PKC activity.

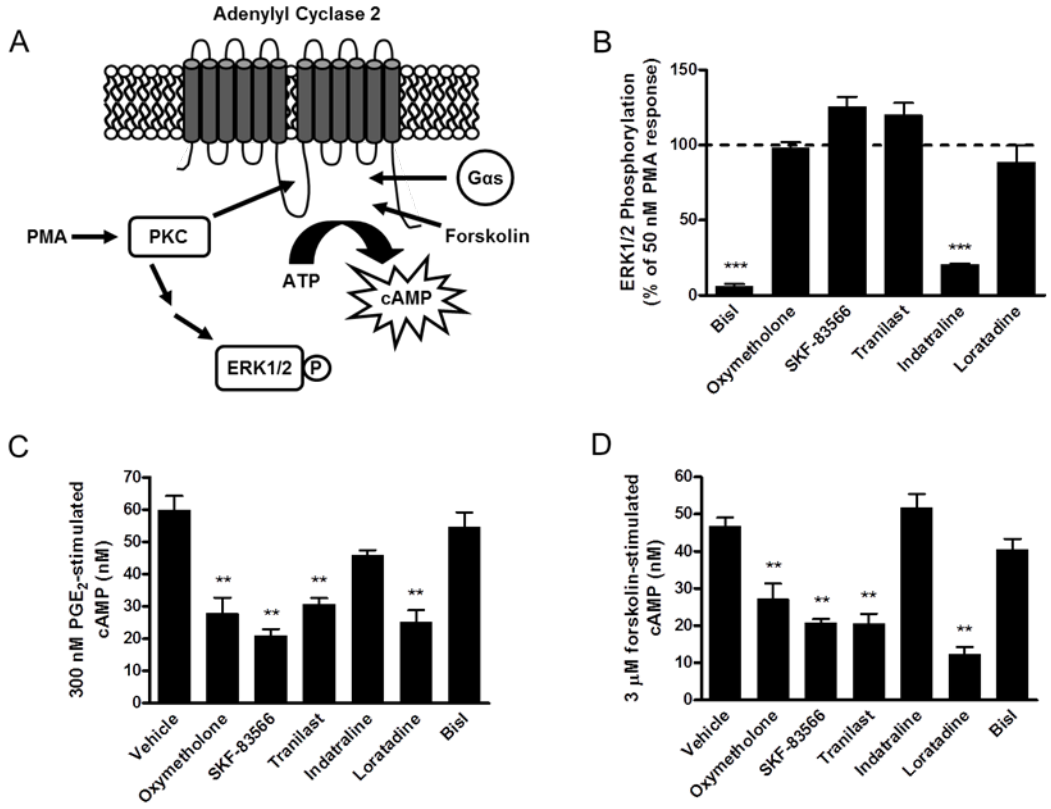

Figure 4.2 Counter-screening for validation of active compounds as AC2 inhibitors. **A.** Schematic for PKC-dependent and PKC-independent activation of AC2**. B.** The effects of test compounds (30 µM) on PMA-stimulated ERK1/2 phosphorylation were measured in HEK-hAC2 cells. Data are mean±S.E.M. of three independent experiments. ***, $p < 0.001$ (one sample *t*-test compared to 100). **C.** The effects of test compounds (30 µM) on 300 nM PGE2-stimulated cAMP accumulation and **D.** 3µM forskolin-stimulated cAMP accumulation was measured in HEK-hAC2 cells. Data are mean±S.E.M. of three independent experiments. ** $p < 0.01$, compared to vehicle condtion, one-way analysis of variance followed by Dunnett's post hoc test.

4.3.3 AC isoform selectivity profiles

The selectivity profiles of the active compounds for inhibition of AC isoforms was explored using intact cell cAMP assays. The ability of compounds to modulate cAMP levels in HEK-hAC2 cells was compared to that of HEK-hAC1 and HEK-hAC5 cells, as well as HEK-wt cells (Figure 4.3). AC1 and AC5 were chosen as representative ACs from group I and group III ACs, respectively. AC1 activity was selectively activated by the calcium ionophore A23187 (3 μM) and AC5 was stimulated by 300 nM forskolin in HEK293 cells stably expressing each isoform (data not shown). Test compounds (30 μM) were evaluated for the ability to modulate selective activation of AC1 and AC5 activity in HEK293 cells. None of the test compounds inhibited AC1 activity, however, loratadine significantly potentiated A23187-stimulated cAMP by ~150%. Studies with HEK-hAC5 cells revealed that loratadine and tranilast strongly inhibited forskolin-stimulated cAMP in HEK-hAC5 cells, while SKF-83566 had more modest activity (~35% inhibition). Tranilast and loratadine also significantly inhibited forskolin-stimulated AC activity in HEK-wt cells, whereas SKF-83566 had no significant effect. Oxymetholone modestly, but significantly inhibited PMA-stimulated cAMP in HEK-hAC2 cells, but had no effect on the AC responses in HEK-hAC1, -hAC5, or –wt cells. These results suggest that the active compounds show distinct patterns of cAMP modulation in HEK293 cells stably expressing recombinant AC1, AC2, or AC5.

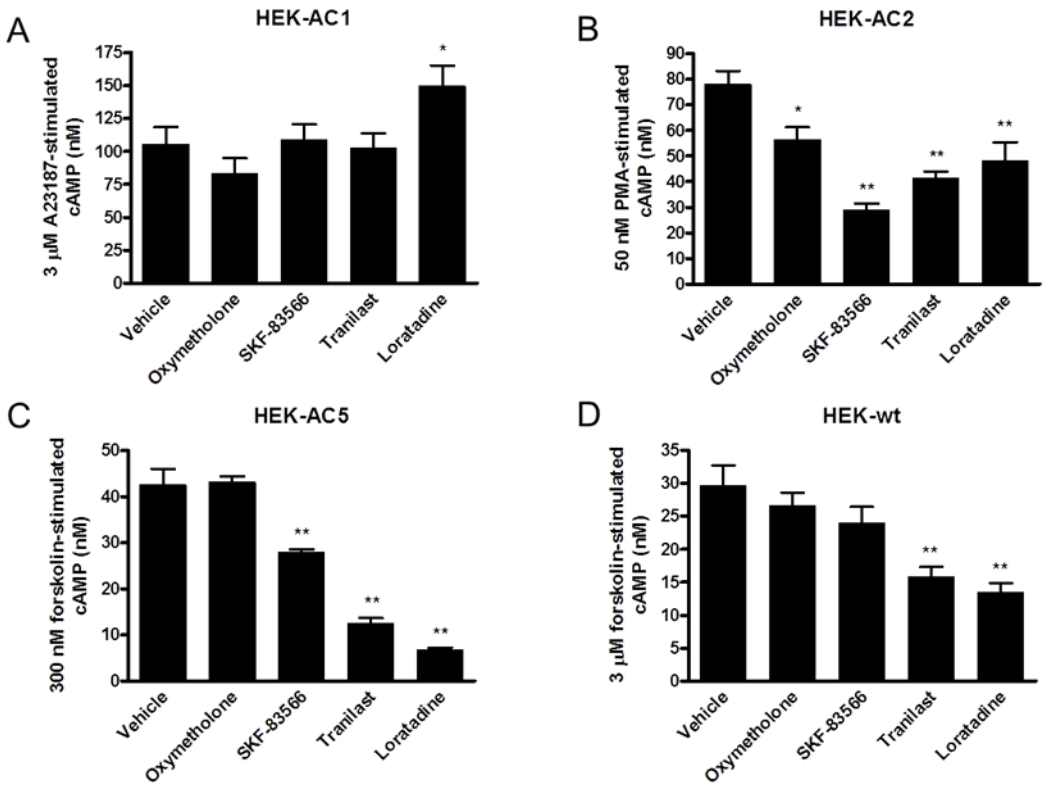

Figure 4.3 AC isoform-selectivity profile of test compounds in intact-cell studies. AC isoform selectivity was assessed by testing the ability of test compounds (30 µM) to modulate **A.** 3µM A23187-stimulated cAMP in HEK-hAC1 cells, **B.** 50 nM PMA-stimulated cAMP in HEK-hAC2 cells, **C.** 300 nM forskolin-stimulated cAMP in HEK-hAC5 cells, and **D.** 3 µM forskolin-stimulated cAMP in HEK-wt cells. * $p < 0.05$, ** $p < 0.01$, compared to vehicle condition, one-way analysis of variance followed by Dunnett's post hoc test.

The direct modulation of AC isoforms was explored using a cell free, reconstituted system to directly assess the effects of test compounds on AC activity (Conley et al., *under review*). Specifically, the effects of the test compounds have been studied in AC activity assays that were performed using membranes from Sf9 insect cells expressing recombinant AC1, AC2, or AC5. As expected, SKF-83566 significantly inhibited forskolin-stimulated AC2 activity (> 40%), suggesting a direct mode of inhibition (mechanistic studies suggest noncompetitive inhibition with respect to ATP). SKF-83566 was inactive against AC1 or AC5. A similar pattern of AC isoform selectivity was observed with tranilast (See figure 4.4 for chemical structures of SKF-83566 and tranilast). In contrast to SKF-83566 and tranilast, a commercially available AC inhibitor, NKY80, showed marked inhibition of AC1 and AC5, but only modest inhibition of AC2 activity. These studies identified SKF-83566 as the most favorable compound to carry into further studies, as it provided the most robust AC2 inhibition, while having no effect on forskolin-stimulated cAMP levels in HEK-wt cells and retention of AC2 inhibition *in vitro*.

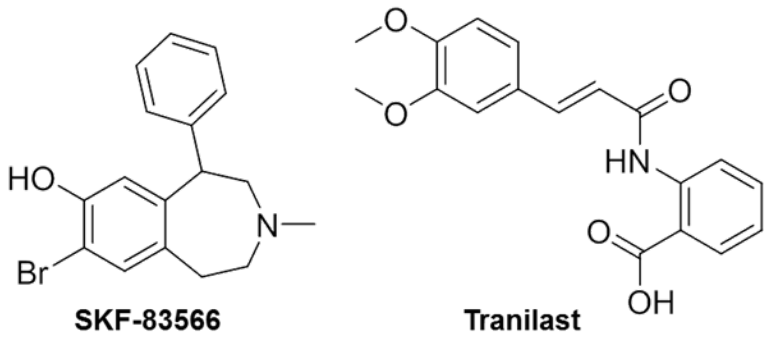

Figure 4.4 Chemical structures of SKF-83566 and tranilast.

The AC isoform-selectivity of SKF-83566 was further characterized by performing a concentration-response analysis for inhibition of cAMP in HEK-hAC2 and HEK-hAC5 cells. SKF-83566 fully inhibited PMA-stimulated cAMP in HEK-hAC2 cells with an IC_{50} value of 10±1.4 µM and maximum inhibition of 104±2% (Figure 4.5A). Additionally, the potency and efficacy values of SKF-83566 for inhibition of 3 µM forskolin stimulation (IC_{50}: 19±3.3 µM and maximum inhibition: 113±2%, n = 3) and 300 nM PGE_2 stimulation (IC_{50}: 21±4.5 µM and maximum inhibition: 117±2%, n = 3) in HEK-hAC2 cells were similar to those observed for inhibition of the PMA response in HEK-hAC2 cells. The robust inhibition below basal levels presumably reflects inhibition of the constitutive AC2 activity. As anticipated from the single point studies, SKF-83566 only partially inhibited forskolin-stimulated cAMP in HEK-hAC5 cells (< 40%) at a concentration of 130 µM. We consistently observed less inhibition of AC5 at 400 µM suggesting a biphasic response (Figure 4.5A). Nonetheless, these results indicate marked selectivity of SKF-83566 for inhibition of AC2 over AC1 and AC5.

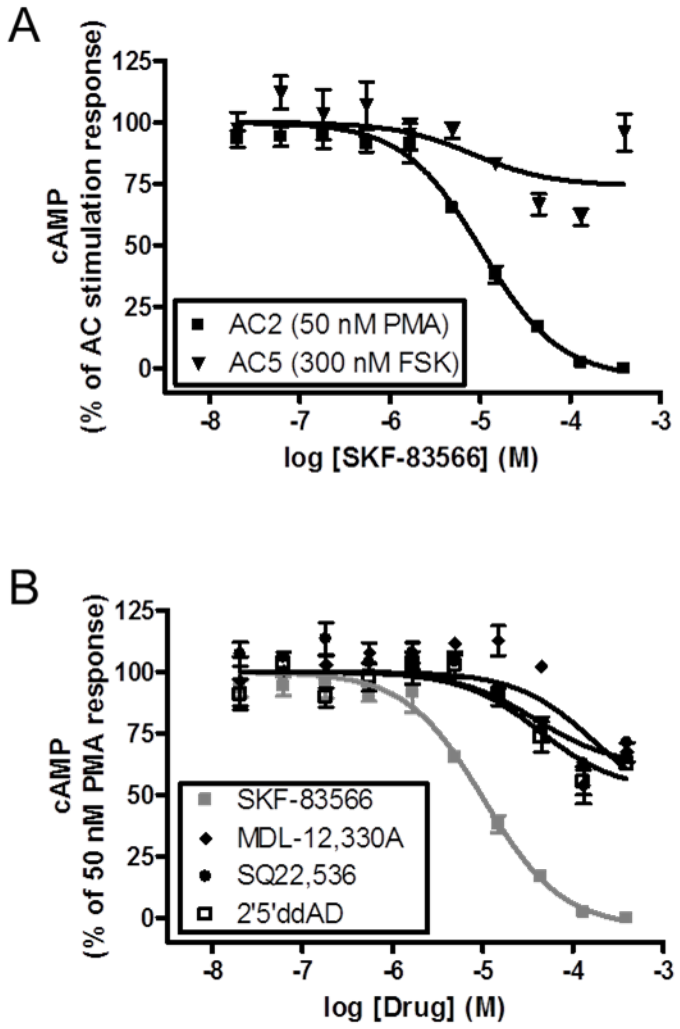

Figure 4.5 Concentration-response analysis of SKF-83566 for inhibition of cAMP. **A.** Dose-response curves of SKF-83566 for inhibition of PMA-stimulated (50 nM) cAMP in HEK-hAC2 cells and forskolin-stimulated (300 nM) cAMP in HEK-hAC5 cells. **B.** Dose-response curves of SKF-83566, MDL-12,330A, SQ22,536, and 2’5’-dideoxyadenosine (2’5’ddAd) for inhibition of PMA-stimulated (50 nM) cAMP in HEK-hAC2 cells. The SKF-83566 data in panels A and B are from the same experiments, as these studies were performed simultaneously. Data are expressed as a percentage of the stimulation response and are reported as the mean±S.E.M. of three independent experiments.

The ability of SKF-83566 to inhibit PMA-stimulated AC2 activity was directly compared to several known AC inhibitors (i.e., SQ22,536, MDL-12,330A, and 2’5’-dideoxyadenosine; Figure 4.5B). Efficacy comparisons revealed marked differences between SKF-83566 (> 100% inhibition) and the known inhibitors (i.e., SQ22,536, 29±3%; MDL-12,330A, 33±4%; and 2’5’-dideoxyadenosine, 38±3%) each at a concentration of 400 µM. These results demonstrate that SKF-83566 displays superior potency and efficacy for inhibition of AC2 activity when directly compared to SQ22,536, MDL12,330A, and 2’5’-dideoxyadenosine in HEK-hAC2 cells.

4.3.4 SKF-83566 as a chemical probe for AC2 function

The most potent and selective AC2 inhibitor (SKF-83566) was examined in cell models where AC2 is natively expressed (along with other AC isoforms), allowing confirmation of its activity in a more physiological context and evaluation of its use as a tool to probe AC2 function. AC2 is reported to be abundantly expressed in skeletal muscle tissues (Ludwig and Seuwen, 2002; Suzuki et al., 1998; Torgan and Kraus, 1996). Therefore, the ability of SKF-83566 to inhibit forskolin-stimulated cAMP accumulation was studied in differentiated mouse C2C12 skeletal muscle myotubes (Figure 4.6). As expected, SKF-83566 inhibited forskolin-stimulated cAMP with an IC_{50} value of 15±6.5 µM and maximum inhibition of 69±8.8%, consistent with SKF-83566 inhibiting endogenous AC2 activity (Figure 4.6).

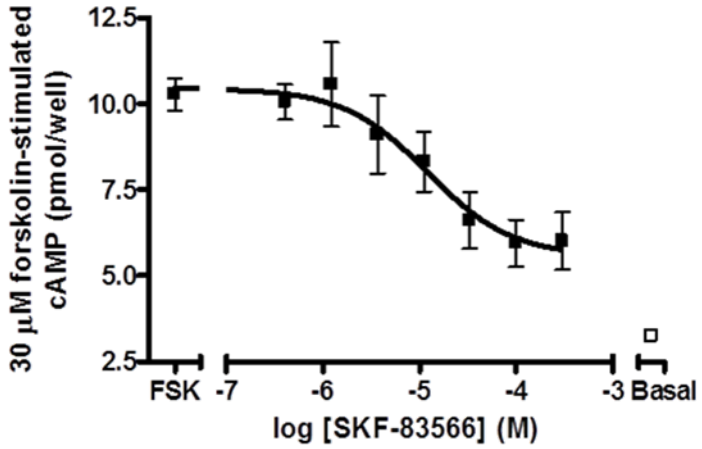

Figure 4.6 SKF-83566 as a chemical probe for native AC2 activity. The effect of SKF-83566 on forskolin-stimulated (30 µM) cAMP was measured in mouse C2C12 skeletal muscle cells that were differentiated into myotubes. Data are mean±S.E.M. of three independent experiments.

Human bronchial smooth muscle cells express AC2, AC4, and AC6 (Bogard et al., 2012) and recent studies suggest that forskolin-stimulated interleukin-6 (IL-6) expression in hBSMCs is selectively mediated by AC2 (Bogard and Ostrom, 2013). Therefore, the effect of SKF-83566 on forskolin-stimulated IL-6 mRNA expression was measured in hBMSCs using quantitative RT-PCR (Conley et al., *under review*). SKF-83566 treatment reduced the forskolin-stimulated IL-6 mRNA to 38±11% of the vehicle treated cells. These results indicate that SKF-83566 inhibited the AC2-mediated upregulation of IL-6 mRNA expression in hBSMCs, suggesting that SKF-83566 may be a useful tool to assess the function of AC2.

4.4 Discussion

Several studies have implicated AC isoforms in physiological functions and disease states, leading to the hypothesis that ACs are potentially novel therapeutic targets (Pierre et al., 2009; Sadana and Dessauer, 2009). However, further research is required to validate AC isoforms as therapeutic targets and advancements have been limited due to the current paucity of small molecule modulators that are potent and AC-isoform selective. The need for additional small molecule tools to assess the in vivo activity of AC isoforms is further reinforced in the case of AC2, where there is both a lack of selective small molecule modulators and an absence of published transgenic or knockout mouse

studies for AC2. Therefore, we developed an HTS-compatible intact-cell small molecule screening approach and subsequent validation paradigm for the discovery of AC2 inhibitors.

The present study used the HTRF cAMP detection technology from Cisbio to develop a robust and scalable HTS assay. The development of an intact-cell assay for small molecule AC modulators was designed to reduce cell-permeability issues that have plagued the utility of several small molecule AC modulators (Seifert et al., 2012). However, the intact-cell screening format presents several challenges that are associated with apparent reductions in the cAMP signal that are independent of direct AC inhibition, including fluorescence detection artifacts, cell death/toxicity, and indirect modes of cAMP reduction that require subsequent validation experiments. The effectiveness of the validation experiments to identify PKC inhibitors was evident in the case of indatraline, as it was found to inhibit PMA-stimulated ERK1/2 phosphorylation, but unable to modulate PKC-independent stimulation of cAMP in HEK-hAC2 cells. The complementary cAMP studies in wild type and stably transfected HEK293 cells validated that the compounds selectively inhibit the exogenously expressed AC isoform. For example, SKF-83566 appeared to have activity for inhibition of cAMP in HEK-hAC2 and HEK-hAC5 cells, but no activity for the inhibition of cAMP in the HEK293 cell background. Conversely, the inhibition observed by tranilast and loratadine was more difficult to interpret because each of these compounds strongly inhibited the forskolin-stimulated cAMP accumulation in the HEK293 cell background, in addition to their apparent activity at the

exogenously-expressed ACs. Therefore, tranilast and loratadine appeared to have effects on multiple AC isoforms, including endogenous ACs expressed in HEK293 cells. For example, our in vitro studies suggested direct modulation of AC2 by tranilast, but indirect modulation of AC1 and AC5. The identification of SKF-83566 as a selective and direct AC2 inhibitor demonstrated the utility of the screening approach and the complementary validation experiments. The success of this initial study combined with additional optimization for increased assay robustness offers promise for future screening efforts of larger and more diverse chemical libraries.

The identification of SKF-83566 as a selective AC2 inhibitor represents another key contribution of the present report. SKF-83566 was originally reported as an antagonist at D1 dopamine and 5HT2 receptors (Berkowitz et al., 1984; Ohlstein and Berkowitz, 1985). Nevertheless, the differences in potency between the receptor antagonism (Berkowitz et al., 1984; Ohlstein and Berkowitz, 1985) and AC2 inhibition by SKF-83566 (i.e., 0.5-30 nM for dopamine/serotonin receptor antagonism versus ~10 μM for AC2 inhibition), together with our control validation assays, suggested that we were observing AC2 inhibition in HEK-hAC2 cells. Interestingly, a recent study suggests that D1/D5 dopamine receptors are closely linked to AC by ligand similarity (Lin et al., 2013), perhaps suggesting that receptor antagonism and AC2 inhibition may have overlapping chemical requirements. Future studies with SKF-83566 should be focused on enhancing its pharmacological properties including its specificity for AC and selectivity for AC2. For example, we used a racemic mixture of SKF-83566,

therefore, pharmacological studies of the resolved enantiomers is expected to provide enhanced potency for inhibition of AC2. Additionally, classic structure-activity relationship studies would also be useful to identify chemical moieties and functional groups that are important for AC2 inhibition and enhance the specificity and isoform-selectivity for AC2.

Despite the potential drawbacks associated with its receptor antagonism, SKF-83566 remains an important addition to the repertoire of AC modulators. For example, SKF-83566 displayed unmatched potency and efficacy for inhibition of AC2 when directly compared to several commonly used AC inhibitors (i.e. NKY80, MDL-12,330A, SQ22,536, and 2'5'-dideoxyadenosine). SKF-83566 also selectively inhibited AC2 (vs other AC isoforms), perhaps offering advantages over the diterpene analog, BODIPY-forskolin, that non-selectively modulates AC isoforms with a bidirectional modulation profile (i.e., inhibition of AC2 and partial activation of AC1 and AC5) (Erdorf et al., 2011; Pinto et al., 2008). The bidirectional modulation makes it difficult to use BODIPY-forskolin as a chemical probe in systems where multiple AC isoforms are expressed. In contrast, our studies with C2C12 mouse skeletal muscle cells and hBSMCs demonstrated the applicability of SKF-83566 as a tool to assess native AC function in a physiological context, suggesting the possibility for its use as an in vivo probe.

The identification of additional selective AC2 modulators is expected to contribute to the understanding of the physiological roles of AC2. There is currently little direct evidence that suggests AC2 as a therapeutic target, but this may be due to the limited availability of research tools and strategies for studying

AC2. Despite these limitations, AC2 has been associated with several diseases, offering a wealth of opportunities for the use of SKF-83566 as a chemical probe for AC2 function. For example, a potential role for AC2-mediated signaling in skeletal muscle physiology is suggested by the abundant expression of AC2 in adult skeletal muscle (Ludwig and Seuwen, 2002; Suzuki et al., 1998; Torgan and Kraus, 1996) and that increased cAMP signaling is implicated in several aspects of muscle physiology including hypertrophy, muscle repair, regeneration, and functional adaptation (Berdeaux and Stewart, 2012). However, the contribution of individual AC isoforms is not well-understood in these physiological processes (Berdeaux and Stewart, 2012), suggesting that SKF-83566 can be used as a chemical tool to study the contribution of AC2-mediated cAMP signaling to muscle physiology and skeletal muscle pathologies including Duchenne's muscular dystrophy and muscle atrophy associated with cancer, ageing, and AIDS.

Recent studies also suggest a role for AC2 in the airway, as AC2 mediates IL-6 expression in hBSMCs (Bogard and Ostrom, 2013). Consistent with this observation, it observed that SKF-83566 was able to inhibit a forskolin-stimulated IL-6 response in hBSMCs (Conley et al., *under review*). Interestingly, increased IL-6 expression has been detected in asthma patients (Neveu et al., 2010) and IL-6 is thought to play an active role in the pathogenesis of lung diseases such as asthma and chronic obstructive pulmonary disease (Neveu et al., 2010; Rincon and Irvin, 2012). Taken together, it's possible that AC2 is contributing to lung disease pathology by mediating elevated IL-6 in hBSMCs.

Thus, SKF-83566 can be utilized to determine if AC2 mediates the increased IL-6 levels in lung diseases, and if this event contributes to pathogenesis.

AC2 also appears to be involved in neuroendocrine tumors (NETs), as *ADCY2* expression is upregulated in a “malignant cluster” of pancreatic NETs (Duerr et al., 2008) and identified as a component of an upregulated cAMP/protein kinase A/CREB pathway in small intestinal NETs (Drozdov et al., 2011). Further in vitro expression and pharmacological analysis suggested that AC2 may be a functional mediator for upregulation of CREB-regulated transcripts that are associated with proliferation in small intestinal NETs (Drozdov et al., 2011). It is also noteworthy that *ADCY2* expression is inversely correlated with survival in colorectal cancer patients (Yu et al., 2011). The studies described above suggest that AC2 has a potential role in the progression of NETs and colorectal cancer, but it is unclear if the enhanced AC2 expression is pathological, protective, or merely a marker of these disease states. SKF-83566 could be used as a chemical probe to test the functional role of AC2 catalytic activity in these pathophysiological states, and in the case of a causal relationship, suggest therapeutic utility for targeting AC2.

In conclusion, the present report describes the development and implementation of an HTS-capable intact-cell screening assay and subsequent validation strategy to identify small molecule inhibitors of AC2. This initial screening effort identified SKF-83566 as a selective AC2 inhibitor with superior pharmacological properties for selective modulation of AC2 when directly compared to the currently available AC inhibitor

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

This work addressed various aspects of AC2 modulation within the framework of three main research aims: 1.) To test the hypothesis that AGS3 expression influences D_{2L}DR-mediated cAMP signaling mediated via AC1 and AC2. 2.) To test the hypothesis that D_{2L}DR-mediated heterologous sensitization of AC2 is Gβγ subunit-dependent. 3.) To develop and implement a high-throughput screening paradigm for the discovery of small molecule modulators of AC2. The conclusions of this work and its implications for future research are discussed below.

Activator of G protein signaling 3 was first identified in a yeast-based screen for receptor-independent activation of G protein signaling and *in vitro* biochemical studies revealed that AGS3 binds Gαi subunits in the GDP-bound state and serves as a guanine nucleotide dissociation inhibitor (De Vries et al., 2000; Takesono et al., 1999). Such biochemical studies suggested the hypothesis that stabilization of Gαi-GDP by AGS3 prevents the inactivating re-association/rearrangement of the G protein heterotrimer, thus resulting in sustained Gβγ subunit signaling. In addition to the biochemical function of AGS3, several studies have implicated AGS3 as in important mediator of behavioral

responses associated with drugs of abuse and have demonstrated that AGS3 expression is up-regulated in the nucleus accumbens core or prefrontal cortex during withdrawal periods following prolonged ethanol or cocaine self-administration, respectively (Bowers et al., 2008; Bowers et al., 2004). Taken together, the biochemical and *in vivo* studies suggest that AGS3 may contribute to molecular changes associated with drugs of abuse by affecting G protein-mediated AC/cAMP signaling. Consistent with this hypothesis, others have demonstrated that AGS3 expression results in attenuated α2-adrenergic receptor-induced heterologous sensitization of AC in CHO cells (Sato et al., 2004). However, AC isoforms display distinct patterns modulation by G protein subunits, suggesting that AGS3 may differentially modulate G protein signaling through AC isoforms. Given the evidence that AGS3, cAMP signaling pathways, and dopamine systems are each involved in drug addiction (Carlezon et al., 2005; McClung and Nestler, 2003; Nestler, 2001), we studied the effects of AGS3 expression on D_{2L}DR-mediated signaling mediated by individual AC isoforms in HEK293 cells.

We examined the ability of AGS3 to modulate recombinant AC1 or AC2 signaling in HEK293 cells following both acute and prolonged activation of the D_{2L}DR. AGS3 expression modestly enhanced the potency of acute quinpirole-induced D_{2L}DR modulation of AC1 or AC2 activity. Prolonged quinpirole activation of the D_{2L}DR was altered by AGS3 in a manner that promoted desensitization of D_{2L}DR-mediated inhibition of AC1, whereas desensitization of D_{2L}DR-mediated AC2 activation was significantly attenuated. Additionally, AGS3

reduced D_{2L}DR-mediated sensitization of AC1 and AC2 in HEK293 cells. These data suggest that AGS3 is involved in altering G protein-mediated cAMP signaling in a complex fashion that is both effector-specific and dependent on the duration of receptor activation.

The effects of AGS3 on D_{2L}DR-mediated sensitization of AC1 and AC2 are consistent with a mechanism where AGS3 disrupts Gβγ subunit signaling associated with persistent Gi-coupled receptor modulation. Several lines of evidence converge to suggest that AGS3 influences Gβγ-mediated effector signaling. For example, our results are consistent with a previous study that demonstrated the ability of an AGS3 consensus peptide to disrupt signaling mediated by a different Gβγ subunit effector (G protein inwardly rectifying potassium channels) following repeated activation of the D_{2S}DR (Webb et al., 2005). Furthermore, Gβγ subunit sequestering by expression of βARKct inhibited D_{2L}DR-mediated sensitization of both AC1 (Nguyen and Watts, 2005) and AC2 (Chapter 3), suggesting that Gβγ subunit signaling is necessary for D_{2L}DR-mediated sensitization of these isoforms. Interestingly, a recent study that utilized bioluminescence resonance energy transfer demonstrated that AGS3 is able to functionally couple with and dissociate from Gi-coupled receptors in an agonist-dependent manner (Oner et al., 2010). Taken together, these studies suggest the possibility that AGS3 competes with Gβγ subunits for binding with Gαi-GDP, thus selectively blocking Gβγ subunit signaling in an indirect manner via Gαi-AGS3 coupling to receptor. Adding further complexity, the effects of AGS3 on G protein signaling appear to be dependent on the

duration of receptor activation because AGS3 alters persistent receptor activation, but only displays modest or no significant effects for signaling in response to acute receptor activation.

Our subsequent studies from chapter 3 directly examined the Gβγ subunit-dependence of D_{2L}DR-mediated sensitization of AC2 and are therefore relevant to the observed effects of AGS3. The regulatory properties of AC2 provide a means to differentiate the contribution of Gα and Gβγ subunits to sensitization. Specifically, AC2 is conditionally activated by Gβγ subunits and is not directly regulated by Gαi/o subunits (Tang and Gilman, 1991; Taussig et al., 1993b; Taussig et al., 1994). These unique regulatory properties of AC2 allowed for the selective study of Gβγ subunit signaling in sensitization of PKC-stimulated AC2 activity. The observations from chapter 3 suggest that similar to other AC isoforms, Gβγ subunits are necessary for the sensitization of AC2. Specifically, the membrane-localized Gβγ subunit sequestering protein βARKct-CD8 and the cell-permeable peptide inhibitor of Gβγ signaling, QEHA-TAT both attenuated D_{2L}DR-mediated sensitization of AC2. The Gβγ subunit-dependence of D_{2L}DR-mediated sensitization of AC2 is in agreement with our observations from chapter 2, where AGS3 blocked D_{2L}DR-mediated sensitization of AC2, further contributing to the emerging hypothesis that AGS3 alters Gβγ subunit signaling following persistent Gi-coupled receptor activation.

Future studies of the molecular effects of AGS3 expression on receptor-mediated Gβγ subunit expression and translocation/localization may shed light on the mechanisms underlying the influence of AGS3 on cAMP signaling

mediated by AC1 and AC2. The localization and translocation of Gβγ subunits have been studied by fluorescence microscopy upon expression of recombinant fluorescently-labeled G protein subunits (Digby et al., 2008), suggesting that the influence of AGS3 on D_{2L}DR-mediated Gβγ subunit localization can be explicitly studied and in a temporal fashion. The effects of AGS3 on the localization of specific Gβ and Gγ subunit dimer combinations can be similarly studied by bimolecular fluorescence complementation (Digby et al., 2006; Digby et al., 2008; Mervine et al., 2006; Yost et al., 2007). This technology relies on the use of a fluorescent protein that is split into two non-fluorescent fragments. One of the fragments can be fused to Gβ and the complementary fragment can be fused to Gγ, and upon interaction of the tagged Gβ and Gγ subunits, the full fluorescent protein reconstitutes, providing fluorescence. In summary, our data and the observations of others suggest that AGS3 may alter Gβγ subunit signaling in response to persistent Gi-coupled receptor activation. Future studies that utilize fluorescently labeled Gβγ subunits may facilitate the direct examination of the effects of AGS3 on the temporal and spatial localization of Gβγ subunits, perhaps providing insight into the mechanistic details of their effects on AC isoform signaling.

It is clear that Gβγ subunit signaling is necessary for D_{2L}DR-mediated sensitization of AC2 in HEK293 cells. Gβγ subunits directly interact with AC2 in several areas and modulate its catalytic activity in a conditional fashion (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006; Tang and Gilman, 1991; Taussig et al., 1993b; Taussig et al., 1994; Weitmann et al., 2001). However, Gβγ subunits

also directly modulate several downstream effectors including PLCβ2, KIR3.1 potassium channels, and voltage-gated Ca^{2+} channels (Khan et al., 2013; Smrcka, 2008), suggesting that development of sensitization may occur by either direct Gβγ modulation of AC2 or via an indirect Gβγ signaling pathway. Though the pharmacological investigation of downstream Gβγ subunit effectors in chapter 3 did not identify effectors involved in sensitization, several observations support the hypothesis that the development of D_{2L}DR-mediated sensitization of AC2 occurs through indirect Gβγ signaling pathways, rather than Gβγ directly modulating AC2. Specifically, D_{2L}DR-mediated sensitization of AC1 is Gβγ subunit-dependent (Nguyen and Watts, 2005), but is inhibited by Gβγ subunits (Taussig et al., 1993b; Taussig et al., 1994). Furthermore, several intermediate signaling components have been implicated in the development of AC sensitization (Johnston et al., 2002; Varga et al., 2002; Varga et al., 2003; Watts and Neve, 2005). We also observed a complete loss of subsequent D_{2L}DR-mediated potentiation of PKC activation of AC2 following long-term D_{2L}DR activation (Chapter 2). Though D_{2L}DR activation acutely stimulates AC2 conditionally via Gβγ subunits, this observation suggests a loss of Gβγ subunit activation of AC2 following long term D_{2L}DR stimulation. Nonetheless, the hypothesis that AC2 sensitization is facilitated by direct Gβγ subunit binding to AC2 can be directly tested. Similar to the Gβγ localization studies proposed above, fluorescently labeled Gβγ subunits can be monitored by fluorescence microscopy following prolonged D_{2L}DR activation. Furthermore, Rluc-tagged AC2 has been utilized in BRET studies to examine the direct interaction of

fluorescently-tagged proteins with Rluc-AC2 (Baragli et al., 2008; Dupre et al., 2007; Petrin et al., 2011; Rebois et al., 2012). These available research tools suggest that BRET studies of fluorescently-tagged Gβγ subunits and Rluc-AC2 following prolonged D_{2L}DR activation can be used to test the hypothesis that D_{2L}DR-mediated sensitization of AC2 occurs by direct modulation of AC2 by Gβγ subunits.

The observations in chapter 3 provided evidence for Gβγ subunits as mediators of AC2 sensitization, but a comprehensive mechanism remains unsolved. However, several possible mechanisms can be proposed for the development of the enhanced PKC-mediated stimulation of AC2 following long-term D_{2L}DR activation. Previous studies suggest that AC2 shows an activator-selective pattern of heterologous sensitization (Cumbay and Watts, 2001). Specifically, persistent D_{2L}DR activation enhances AC2 cAMP responses to phorbol ester activation of PKC, but not in response to forskolin or the Gs-coupled β-adrenergic receptor agonist, isoproterenol (Cumbay and Watts, 2001). These data clearly implicate PKC in AC2 sensitization. It is possible that long-term D_{2L}DR activation results in enhanced PKC expression in HEK293 cells, leading to enhanced responses to phorbol ester-mediated AC2 activation. PKC expression profiling by western blotting for PKC isoforms in HEK293 cells can be performed following prolonged quinpirole treatment and compared to the expression levels of a vehicle treatment. PKCα (Zimmermann and Taussig, 1996) and PKCδ (Nguyen and Watts, 2006) are implicated in the acute activation of AC2, but a comprehensive assessment of the effects of PKC isoforms on AC2

remains to be reported. A combination of approaches that include siRNA, PKC isoform overexpression, and isoform-selective PKC peptide inhibitors can be used to determine the specific PKC isoforms that are involved in both acute and subsequent activation of AC2 following persistent D_{2L}DR activation. It is also possible that long-term D_{2L}DR activation altered the levels of PKC that are in close proximity to AC2. The latter possibility could be mediated by two anchoring proteins (i.e., RACK1 and AKAP79) that are endogenously expressed in HEK293 cells (Bauman et al., 2006; Liu et al., 2007). For example, the PKC anchoring protein RACK1 functions to target PKC to its substrates (Schechtman and Mochly-Rosen, 2001). Furthermore, RACK1 is known to directly bind Gβγ subunits and influence Gβγ-mediated signaling (Chen et al., 2008; Chen et al., 2005; Chen et al., 2004). Alternatively, AKAP79 directly interacts with AC2 (Efendiev et al., 2010) and is also known to scaffold PKA and PKC (Welch et al., 2010), but Gβγ subunit-modulation of this protein has not been reported (based on my knowledge). Future research should be focused on the potential role of these proteins in the development of D_{2L}DR-mediated sensitization of AC2.

Molecular studies of heterologous sensitization have largely focused on the roles G proteins (e.g., Gαi/o, Gβγ, and Gαs subunits), proteins that modulate G protein signaling (e.g., RGS and AGS proteins), and protein kinases that are well-known to be downstream of G proteins. The precise mechanisms of AC sensitization are not well understood, even after nearly four decades of research. We propose that unbiased approaches can be utilized to identify novel components of the signaling pathways that are involved in the mechanisms of AC

sensitization. Such unbiased approaches include siRNA library screening or small molecule screening of known pharmacological modulators (e.g., kinase inhibitor collections). The cell-based cAMP detection methodology that our laboratory has utilized to study sensitization, however, was generally unsuitable for siRNA library or small molecule screening endeavors. Therefore, steps were taken to reduce the number of wash and decant steps, miniaturize the cAMP quantification assay, and identify an assay workflow that is amenable to automation to facilitate the development an assay format that is amenable to the unbiased strategies for the study of sensitization (Conley et al., *in press*). As part of the initial validation of the high-throughput screening capable cAMP assay and workflow, the implementation of this technology to screen for small molecule inhibitors of AC2 was reported in chapter 4. The cell-based high-throughput screening paradigm that we developed can likely be readily adjusted to measure small molecule modulation of heterologous sensitization. Furthermore, the assay format has since been modified to accommodate screening of an siRNA library against the kinome for inhibition of Gi-coupled receptor-mediated sensitization of AC isoforms (Conley et al., *in press*). It is expected that these unbiased approaches will provide new mechanistic information regarding the adaptive heterologous sensitization response.

The development of an HTS-capable cell-based assay to measure the modulation of AC activity was initially designed to address assay format requirements for the unbiased approaches to study heterologous sensitization as described above. However, as a strategic intermediate step to the development

of an assay format for an siRNA library screen for the modulation of heterologous sensitization of AC, we first developed and implemented a screening assay for small molecule modulators of acute AC activity. This strategy allowed for the initial development of a relatively simpler assay, while providing the opportunity to assess the robustness of the cAMP detection technology and the miniaturized assay platform for its HTS-amenable attributes. Furthermore, the lack of potent and selective small molecule AC modulators presented an opportunity to address a well-known research need in the AC field, while providing proof-of-concept for the HTS-amenable cAMP assay. Specifically, AC isoforms are implicated in several physiological processes and disease states (Pierre et al., 2009; Sadana and Dessauer, 2009), but advancements in the therapeutic targeting of AC isoforms have been limited by the lack of potent and isoform-selective small molecule modulators (Pavan et al., 2009; Pierre et al., 2009; Seifert et al., 2012). The discovery of AC isoform-selective small molecules is expected to facilitate the validation of AC isoforms as therapeutic targets and augment the study of AC isoform function *in vivo*. Identification of chemical probes for AC2 is particularly important because there are no published genetic deletion studies and few small molecule modulators. Chapter 4 demonstrates the development and implementation of an intact-cell small molecule screening approach and subsequent validation paradigm for the discovery of AC2 inhibitors. The NIH clinical collections I and II were screened for inhibitors of AC2 activity, using PMA-stimulated cAMP accumulation as a functional readout. Active compounds were subsequently confirmed and validated as direct AC2 inhibitors using

orthogonal and counter screening assays. The screening effort identified SKF-83566 as a selective AC2 inhibitor with superior pharmacological properties for selective modulation of AC2 when compared to currently available AC inhibitors. Furthermore, the utility of SKF-83566 as a small molecule probe to study the function of endogenous ACs was demonstrated in C2C12 mouse skeletal muscle cells.

Future studies should be aimed at understanding the molecular basis of AC2 inhibition by SKF-83566. A combination of biochemical, molecular modeling, and mutagenesis studies have typically been employed to understand the mechanisms of small molecule modulation of AC. Biochemical studies of enzyme kinetics using cell membranes that overexpress AC2 suggest that SKF-83566 is noncompetitive with respect to ATP (Conley et al., *under review*), but its relationship to other activators, such as forskolin, or Gαs (Dessauer, 2002; Dessauer and Gilman, 1997; Dessauer et al., 1999; Iwatsubo et al., 2004; Onda et al., 2001) can also be studied. There is also a precedent for molecular modeling based on the crystal structures of the catalytic C1 and C2 domains (Pinto et al., 2008; Tesmer et al., 1997; Zhang et al., 1997). Similar molecular modeling analyses are underway for SKF-83566 docking to AC to predict the specific site of interaction. Subsequent to molecular modeling studies, mutation of residues that are predicted to be crucial for binding can be performed to experimentally validate the model. The complementary approaches described above may provide useful insight into the mechanism of AC2 modulation by SKF-83566.

SKF-83566 is an important new chemical probe for AC2, but it also displays antagonist activity against D1 dopamine and 5HT2 serotonin receptors (Berkowitz et al., 1984; Ohlstein and Berkowitz, 1985). Future efforts should be focused toward strategies to enhance the pharmacological properties of SKF-83566 to increase the specificity for AC and enhance the potency and selectivity for inhibition of AC2. First, our study utilized SKF-83566 as a racemic mixture, warranting additional pharmacological studies to determine which enantiomer is more active for inhibition of AC2. Interestingly, a recent study suggests that D1/D5 dopamine receptors are closely linked to AC by ligand similarity (Lin et al., 2013), perhaps suggesting that receptor antagonism and AC2 inhibition will share the same active enantiomer (i.e, (+)-SKF-83566) (Berkowitz et al., 1984; Ohlstein and Berkowitz, 1985). Furthermore, (+)-SCH-23390 (a structural analogue of SKF-83566) significantly inhibited PMA-stimulated AC2 activity in HEK-hAC2 cells, suggesting that (+)-SKF-83566 is the active enantiomer for inhibition of AC2 (Table A.2 and Figure A.1). The enantiomer studies are expected to at least provide enhanced potency for inhibition of AC2. Additionally, classic structure-activity relationship studies would also be useful to identify chemical moieties and functional groups that are important for AC2 inhibition and enhance the specificity and isoform-selectivity for AC2. A modest structure-activity study is reported in Table A.2 and Figure A.1 of the appendix.

Our results from chapter 4 demonstrate the utility of the screening approach and suggest that the series of complementary post-screening experiments are able to validate direct and selective AC2 inhibitors. Importantly,

the success of the initial pilot screen of a 727 compound collection (NIH clinical collections I and II) suggests that the screening and validation paradigm is sufficiently robust to accommodate future screening of larger and more diverse chemical libraries to identify additional novel small molecule AC2 inhibitors. Consistent with this prediction, a screen of the Spectrum Collection (2,320 compounds) for inhibitors of AC2 activity has been performed and the results and initial confirmation of activity is reported in the appendix (Table A.3 and Table A.4). Furthermore, the assay format that was developed to study cAMP levels in HEK-hAC2 cells is expected to be readily adapted for other AC isoforms, including AC1 and AC5. Specifically, parameters such as pharmacological stimulation conditions and cell number should be optimized for each specific target, but the overall semi-automated workflow and cAMP detection technology is expected to support these studies without modification. Identification of new small molecule modulators of AC1 and AC5 are expected to contribute to our understanding of these isoforms in physiological processes including pain perception, learning and memory, and cardiac function.

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APPENDIX

APPENDIX

A high-throughput screening approach for the discovery of small molecule inhibitors of AC2 was described in chapter 4. Specifically, the NIH clinical collections I and II were screened for the ability of compounds (25 μM) to inhibit 50 nM PMA-stimulated AC2 activity in HEK-hAC2 cells. The complete screening results are reported in Table A.1.

Table A.1 Screening of NIH Clinical Collections I and II for inhibition of AC2 activity. The NIH Clinical Collections I and II (25 µM) were screened for inhibition of PMA-stimulated (50 nM) cAMP accumulation in HEK-hAC2 cells using the Cisbio HTRF cAMP dynamic 2 detection methodology. The data represent the average percent inhibition of the PMA-stimulated cAMP response from duplicate plates.

Compound Name	**% Inhibition**
(-)-Cotinine	-14.29
(+)-3-HYDROXY-N-METHYLMORPHINAN D-TARTRATE	23.30
(+)-CIS-DILTIAZEM HYDROCHLORIDE	-60.31
(+/-)-Epinephrine hydrochloride	-123.98
(+/-)-NOREPINEPHRINE HYDROCHLORIDE	-71.99
(Â±)-Vesamicol hydrochloride	-5.49
1-(2-Methyl-5-nitro-imidazol-1-yl)-propan-2-ol	-41.71
1,1-DIMETHYL-4-PHENYLPIPERAZINIUM IODIDE	-12.35
1,3,5(10)-ESTRATRIEN-3-OL-17-ONE SULPHATE, SODIUM SALT	-34.06
10H-Phenothiazine, 2-chloro-10-[3-(4-methyl-1-piperazinyl)propyl- [CAS]	35.21
17-BETA-ESTRADIOL 17-VALERATE	-427.70
19-Norethindrone	-79.01
19-NORETHINDRONE ACETATE	-162.15
1-BENZYLIMIDAZOLE	23.73
1H-Cyclopenta[b]quinolin-9-amine, 2,3,5,6,7,8-hexahydro-, monohydrochloride- [CAS]	-1.71
1H-Imidazol-2-amine, N-(2,6-dichlorophenyl)-4,5-dihydro- [CAS]	-14.30
1H-Imidazole-5-carboxylic acid, 1-(1-phenylethyl)-, ethyl ester, (R)- [CAS]	-43.54
1H-Indole-2-propanoic acid, 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-Alpha,Alpha-dimethyl-5-(1-methylethyl)- [CAS]	-866.27
2(1H)-Pyrimidinone, 4-amino-1-ÃƒÂ¿-D-arabinofuranosyl- [CAS]	11.06
2-(2-AMINOETHYL)PYRIDINE	2.82
2',3'-DIDEOXYCYTIDINE	-16.86
2',3'-DIDEOXYINOSINE	-21.84
2-CHLORO-2'-DEOXYADENOSINE	-129.30
2-CHLOROADENOSINE	-213.14
2H-Indol-2-one, 1,3-dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)- [CAS]	-95.83
3(2H)-Pyridazinone, 6-[4-(difluoromethoxy)-3-methoxyphenyl]- [CAS]	-210.56
3,5,3'-TRIIODOTHYRONINE	-11.34
3-[3,5-DIBROMO-4-HYDROXYBENZOYL]-2-ETHYLBENZOFURAN	-147.33
3'-deoxydenosine	-10.67
3-HYDROXY-1,2-DIMETHYL-4(1H)-PYRIDONE	-0.82
3-Hydroxy-2-methyl-4-pyrone	-61.63
3-PYRIDINEMETHANOL	36.49
4-(AMINOMETHYL)BENZENESULFONAMIDE ACETATE	-57.21
4-Chloro-N-(2-morpholin-4-yl-ethyl)-benzamide	-20.07

Table A.1 Continued.

4-Thiazolidinecarboxylic acid, 2-oxo-, (R)- [CAS]	-28.68
5-Amino-2-hydroxy-benzoic acid	11.79
5-Azacytidine	-26.30
5-fluoro-2-pyrimidone	-1.08
5-FLUOROCYTOSINE	13.73
5-FLUOROURACIL	-4.98
5-Methoxytryptamine	-35.57
5-Nonyloxytryptamine	-39.73
6-[2-ETHOXY-1-NAPHTHAMIDO]-PENICILLIN SODIUM SALT	-65.07
6ALPHA-METHYL-11BETA-HYDROXYPROGESTERONE	-102.01
6-AMINOINDAZOLE	-10.70
6-AZAURIDINE	-3.55
6H-Pyrido[2,3-b][1,4]benzodiazepin-6-one, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro- [CAS]	6.14
7-NITROINDAZOLE	-49.80
8-Azaspiro[4.5]decane-7,9-dione, 8-[2-[[(2,3-dihydro-1,4-benzodioxin-2-yl)methyl]amino]ethyl]-, monomethanesulfonate [CAS]	-28.35
8-Chloro-11-piperidin-4-ylidene-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridine	13.56
9-AMINO-1,2,3,4-TETRAHYDROACRIDINE HYDROCHLORIDE	11.92
ACARBOSE	-10.38
ACEBUTOLOL HYDROCHLORIDE	-25.27
Acetamide, 2-amino-N-(1-methyl-1,2-diphenylethyl)-, (+/-)- [CAS]	-36.70
Acetazolamide	-48.41
Acitretin	-23.36
ACTARIT	-7.99
Acyclovir	-30.21
Adenine 9-beta;-D-arabinofuranoside	-9.90
Adenosine, N-(2-hydroxycyclopentyl)-, (1S-trans)- [CAS]	-36.70
Albalon	-71.39
Albendazole	-62.52
ALFUZOSIN	-1.64
Allegra	-10.48
Allopurinol	-43.46
ALOSETRON HCl	-42.93
Alprazolam	-46.53
Altanserin	-611.22
Altretamine	-12.49
AM 404	-283.12
AM-251	5.15
AMCINONIDE	-1.11
AMFEBUTAMONE HCl	-12.60
Aminoglutethimide	-38.97
Aminolevulinic Acid	-80.40

Table A.1 Continued.

AMIODARONE HYDROCHLORIDE	-370.47
Amisulpride	-19.13
AMLEXANOX	41.47
AMLODIPINE BASE	24.13
AMOXAPINE	-16.78
AMOXICILLIN CRYSTALLINE	-60.10
Ampicillin Sodium	-64.53
AMPIROXICAM	-39.71
Anafranil	-68.51
ANAGRELIDE HCl	-115.28
ANASTROZOLE	-9.63
Annoyltin	-15.14
ARGATROBAN	-1.41
ARIPIPRAZOLE	-54.30
Artane	1.60
ARTEMETHER	-53.69
ARTESUNATE	-12.63
ATENOLOL	14.86
ATOMOXETINE HCl	1.54
ATRACURIUM BESYLATE	-64.73
ATROPINE	-60.91
Azasetron	-2.06
Azathioprine	-29.00
AZELASTINE HCl	1.65
Azithromycin	-44.56
BALSALAZIDE	-16.93
Beclomethasone	-66.70
Beclomethasone dipropionate	-20.74
BENACTYZINE HYDROCHLORIDE	-48.45
BENAZEPRIL HCl	-4.13
Bendrofluazide	-24.41
BENIDIPINE HCl	-194.19
BENPROPERINE PHOSPHATE	-8.36
Bentyl	-89.31
Benzeneacetic acid, 2-[(2,6-dichlorophenyl)amino]-, monosodium salt [CAS]	-2.76
Benzeneacetic acid, Alpha-(hydroxymethyl)-, 9-methyl-3-oxa-9-azatricyclo[3.3.1.02,4]non-7-yl ester, [7(S)-(1Alpha,2ÃƒÂ¿,4ÃƒÂ¿,5Alpha,7ÃƒÂ¿)]- [CAS]	-14.01
Benzeneacetonitrile, Alpha-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-Alpha-(1-methylethyl)-, (R)- [CAS]	-19.11
Benzenebutanoic Acid	-5.73

Table A.1 Continued.

Benzo[a]phenanthridine-10,11-diol, 5,6,6a,7,8,12b-hexahydro-, trans-[CAS]	-0.81
BESTATIN	-23.10
BETA-ESTRADIOL	-61.63
Betamethasone	-16.61
Betaxolol hydrochloride	9.57
BICALUTAMIDE	-55.60
Bifemelane	-15.19
BIFONAZOLE	-269.21
BISOPROLOL FUMARATE	-25.29
Brimonidine	-86.58
Brucine	9.29
BUDESONIDE	-26.53
Buflomedil HCl	-6.25
BUMETANIDE	-1.04
BUPROPION HYDROCHLORIDE	-14.45
Buspar	-25.47
Busulfan	-12.09
CALCIPOTRIOL	-78.88
CALCITRIOL	-79.52
Cantil	4.85
Capsaicin	-58.39
CAPTOPRIL	-35.91
Carbamazepine	-47.13
CARBIDOPA	-14.95
Carbinoxamine Maleate	-52.66
Cardene	-391.56
Carisoprodol	-16.15
CARMOFUR	-5.31
Carvedilol	-85.91
CCPA	-32.69
CEFACLOR	-6.56
CEFATRIZINE PROPYLENE GLYCOL	-28.72
CEFAZOLIN SODIUM SALT	-44.82
CEFDINIR	10.10
CEFIXIME TRIHYDRATE	-6.49
CEFOTAXIME SODIUM SALT	-28.23
CEFOXITIN SODIUM SALT	-58.42
CEFPODOXIME PROXETIL	-42.62
Cefuroxime	9.32
Celecoxib	16.11
Cephalexin monohydrate	-47.87
CERIVASTATIN Na	-49.49
Cetirizine	-59.23

Table A.1 Continued.

CETRAXATE HCl	-19.18
CGS 12066B	-235.66
CGS 15943	-118.86
CHLORAMBUCIL	-54.68
Chloramphenicol	-31.91
CHLORDIAZEPOXIDE	-109.83
Chlorothiazide	8.06
Chloroxine	-302.28
Chlorpheniramine	-4.76
Chlorpropamide	-74.63
Chlorthalidone	-2.90
Chlorzoxazone	-31.15
CILASTATIN Na	11.23
Cimetidine	-17.64
Cinanserin	-28.54
Cisapride	-45.26
Citalopram	-86.53
citalopram hydrobromide	-51.02
CLARITHROMYCIN	0.08
Clobenpropit	-37.96
CLOBETASOL PROPIONATE	-82.01
CLOFAZIMINE	-76.90
Clomid	-266.53
clopidogrel	-69.38
CLOTRIMAZOLE	-136.54
Clozapine	-22.10
Cogentin Mesylate	-36.32
Cortell	-62.85
CORTICOSTERONE	-74.11
CORTISONE	-78.06
CORTISONE ACETATE	-39.61
Cromolyn Sodium	0.10
CYPROHEPTADINE	-26.41
Cytoxan	-21.75
d-3-Methoxy-N-methylmorphinan hydrobromide	-21.95
DACTINOMYCIN	-28.81
DANAZOL	-351.79
Dantrolene sodium salt	-16.00
Dapsone	-5.46
DAUNORUBICIN HYDROCHLORIDE	-18.55
D-CYCLOSERINE	-10.81
D-CYCLOSERINE	-42.47
DEHYDROCHOLIC ACID	21.82
DEHYDROEPIANDROSTERONE	-50.60

Table A.1 Continued.

DELTA1-HYDROCORTISONE 21-HEMISUCCINATE SODIUM SALT	-5.61
Demeclocycline	-45.09
DEPO-MEDROL	-48.24
DEPRENALIN	-45.50
DEPRENALIN	-74.72
DESOXIMETASONE	-16.70
Dexamethasone	-13.53
DEXBROMPHENIRAMINE MALEATE	-27.93
DEXCHLORPHENIRAMINE MALEATE	27.54
Diazepam	-43.54
DIAZOXIDE	-9.39
Dibenzyline	-459.58
DICHLOROACETIC ACID	-55.71
DICLOXACILLIN SODIUM	-52.25
Diflunisal	-144.95
DIGOXIN	35.26
Dilantin	-12.65
Diphenhydramine hydrochloride	4.15
DIPHENOXYLATE	-65.13
diphenylcyclopropenone	-424.00
DIPYRIDAMOLE	15.69
Disipal	0.76
DL-PENICILLAMINE	-50.23
DOCETAXEL	-34.98
DOFETILIDE	-13.63
DOLASETRON MESYLATE	-29.16
Donepezil	-26.59
DOXAPRAM HYDROCHLORIDE	-14.91
Doxazosin	-92.28
Doxepin	-51.07
DOXEPIN HYDROCHLORIDE	-26.94
DOXORUBICIN HYDROCHLORIDE	33.53
DOXYCYCLINE	37.36
Doxylamine succinate salt	-27.08
DROPERIDOL	-39.13
Duloxetine	16.04
DULOXETINE HCl	50.54
Duremesin	-458.71
Duvadilan	-18.67
EBSELEN	-166.51
Econazole Nitrate	-301.82
EDROPHONIUM CHLORIDE	46.54
EFAVIRENZ	-44.75
ENALAPRIL MALEATE	-2.74

Table A.1 Continued.

ENALAPRILAT	22.39
ENROFLOXACIN	-26.16
Epigallocatechin gallate	-13.06
EPIRUBICIN HYDROCHLORIDE	18.31
Eryped	4.37
ESCITALOPRAM OXALATE	-33.09
ESMOLOL HYDROCHLORIDE	-9.35
ESOMEPRAZOLE Mg	-70.39
Eszopiclone	-48.05
ETHACRYNIC ACID	-34.64
Ethambutol	-56.84
Ethionamide	-55.49
Ethylestrenol	-81.07
ETHYNYLESTRADIOL	-91.02
Etodolac	-46.54
Etomidate	9.69
etoposide	-16.54
Evista	-100.36
EXEMESTANE	-115.77
EZETIMIBE	-46.48
FAMCICLOVIR	-28.61
FAMOTIDINE	-5.01
Felbamate	-41.46
Felodipine	-95.05
FENOFIBRATE	-228.94
FENOLDOPAM MESYLATE	-22.47
FENPIVERINIUM BROMIDE	5.18
FINASTERIDE	-22.29
Flecainide Acetate	-17.60
Floxuridine	-13.78
FLUBENDAZOLE	-53.87
Fluconazole	-39.05
FLUDARABINE	22.76
Flumadine	-12.51
FLUMAZENIL	-29.89
FluniSOLIDe	-198.75
FLUOCINOLONE ACETONIDE	12.99
FLUOCINOLONE ACETONIDE 21-ACETATE	-35.57
Fluorometholone	-76.25
Fluperlapine	-9.92
Fluphenazine Dihydrochloride	14.21
Flurbiprofen	-44.40
Flurbiprofen	-78.82
FLUTAMIDE	-56.61

Table A.1 Continued.

FLUTICASONE PROPIONATE	29.83
fluvastatin	-44.95
Fluvoxamine	16.65
Folic acid	-22.44
FORMOTEROL FUMARATE DIHYDRATE	-88.12
FTORAFUR	-27.87
FUROSEMIDE	-11.34
Gabexate mesylate	-91.14
Galanthamine	-41.37
Ganciclovir	-41.16
Gatifloxacin	-0.22
GEMFIBROZIL	-44.81
GLIMEPIRIDE	-40.10
GLIPIZIDE	11.22
Glyburide	-5.62
Glycine, N-[2-[(acetylthio)methyl]-1-oxo-3-phenylpropyl]-,phenylmethyl ester [CAS]	-7.71
Glycopyrrolate	8.42
Glycopyrrolate	-29.69
GOSERELIN ACETATE	2.29
GR 89696	17.49
GRANISETRON HCl	-16.86
Griseofulvin	-83.55
Guanidine, N-cyano-N'-(1,1-dimethylpropyl)-N''-3-pyridinyl- [CAS]	9.58
HALOMETASONE MONOHYDRATE	-6.37
Haloperidol	15.22
Hexachlorophene	-257.07
HOMOHARRINGTONINE	-37.36
Homoveratrylamine	-36.42
HONOKIOL	-137.50
HTMT	0.82
HUPERZINE A	17.45
Hydrochlorothiazide	-54.49
HYDROCORTISONE	-39.84
HYDROCORTISONE HEMISUCCINATE	-65.75
HYDROFLUMETHIAZIDE	10.38
HYPEROSIDE	-68.78
IBUPROFEN	-35.67
ICARIIN	-43.84
IDARUBICIN HCl	-58.20
IDEBENONE	-328.07
Ifenprodil	16.10
IMATINIB MESYLATE	-123.01
Imodium	-8.23

Table A.1 Continued.

INDAPAMIDE	-57.29
Indatraline	38.92
Inderal	-14.01
INDINAVIR SULPHATE	1.17
Indirubin	26.64
Indomethacin	-26.12
Intropin	41.82
IPRATROPIUM BROMIDE MONOHYDRATE	-53.51
IPRIFLAVONE	-31.76
IRBESARTAN	-25.62
IRINOTECAN HCl)trihydrate)	-32.70
IRSOGLADINE MALEATE	-30.34
Isoniazid	-6.49
Isoquercitrin	-17.85
Isotretinoin	-150.35
ISRADIPINE	-99.96
Isuprel	-99.87
ITAVASTATIN Ca	-53.39
ITOPRIDE HCl	-35.59
ITRACONAZOLE	-90.04
Kemadrin	-56.49
ketoconazole	-170.07
KETOPROFEN	-81.27
Ketorolac tromethamine	-73.56
KETOTIFEN FUMARATE	-15.38
KITASAMYCIN	-38.50
L-694,247	-3.82
LABETALOL HYDROCHLORIDE	-19.73
LACIDIPINE	-881.61
LAMIVUDINE	-11.89
LAMIVUDINE	-29.52
LAMOTRIGINE	-12.56
LANSOPRAZOLE	-228.54
LATANOPROST	-364.86
LETROZOLE	-57.57
LEVETIRACETAM	-17.12
LEVOCETIRIZINE	-17.88
LEVOFLOXACIN	23.25
LEVONORGESTREL	-46.15
LEVOSULPIRIDE	-37.31
L-Glutamic acid, N-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]- [CAS]	10.82
LIDOCAINE	-21.96
Lincomycin hydrochloride	-66.35

Table A.1 Continued.

LINEZOLID	-10.12
LOBELINE HYDROCHLORIDE	-13.53
LOFEPRAMINE	-179.59
LOFEXIDINE HCl	0.30
LOMERIZINE DiHCl	-512.44
LOMIFYLLINE	-0.91
LORATADINE	64.39
Lorazepam	-221.43
L-Ornithine, N5-[imino(methylamino)methyl]-[CAS]	15.83
LOSARTAN Potassium	-9.59
LOTEPREDNOL ETABONATE	-6.30
Lovastatin	-153.47
Loxapine succinate	-21.33
LOXOPROFEN SODIUM	1.74
L-THYROXINE	-9.99
LY 171883	13.22
Maprotilline HCl	33.13
Maxolon	-47.34
Mebendazole	-62.43
MECILLINAM	-28.98
Meclomen	-129.92
MEDROXYPROGESTERONE	-58.63
Medroxyprogesterone 17-acetate	-58.25
Mefenamic Acid	-141.11
Mefloquine hydrochloride	31.17
MEGESTROL ACETATE	-52.62
Meloxicam	-22.67
MEMANTINE HYDROCHLORIDE	3.22
Memantine hydrochloride	-62.34
MEPIRIZOLE	-33.25
MEPIVACAINE HYDROCHLORIDE	-32.43
Mercaptopurine	-6.31
MEROPENEM	6.18
Mesna	-42.14
Mesoridazine	27.11
Mestanolone	-49.42
Mestinon	15.65
MESTRANOL	-65.51
Metaproterenol	-178.71
Methanesulfonamide, N-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl]-, dihydrochloride [CAS]	-61.27
Methazolamide	-55.00
Methimazole	-56.87
Methocarbamol	-78.32

Table A.1 Continued.

Methoxsalen	-250.02
METHYLANDROSTENEDIOL	-25.46
Methyldopa	-49.37
METHYLPREDNISOLONE	-5.71
Methyltestosterone	-49.64
METRONIDAZOLE	15.03
Metylperon	-41.07
METYRAPONE	-43.67
mevastatin	-44.00
Mexitil	-26.24
MICONAZOLE NITRATE	-1049.78
Micropenin	1.62
MIDAZOLAM HCl	-23.02
MIFEPRISTONE	-47.38
MIGLITOL	-31.50
Milnacipran	1.31
MILRINONE	-2.02
MINOCYCLINE HYDROCHLORIDE	-48.09
Minoxidil	-42.84
Miochol	-19.44
MIRTAZAPINE	-63.94
mirtazapine	-21.76
MITOXANTRONE	85.56
Moban	-6.90
Modafinil	-28.40
MONTELUKAST Na	-35.55
MOSAPRIDE CITRATE	-35.42
MOXIFLOXACIN HCl	22.06
MOXONIDINE HCl	-2.44
Mupirocin	-79.38
N,N'-DIACETYL-1,6-DIAMINOHEXANE	-19.56
NABUMETONE	-16.27
NADOLOL	-6.63
Nafadotride	8.12
NAFTOPIDIL	-115.20
Nalbuphine	-38.64
Nalidixic Acid	-16.93
NALOXONE HYDROCHLORIDE	-48.66
NALTREXONE HYDROCHLORIDE	-7.68
Naltrindole	-16.75
nandrolone	-1195.60
NAPROXEN SODIUM	-101.28
NATEGLINIDE	-2.38
Nefazodone	-131.94

Table A.1 Continued.

NELFINAVIR MESYLATE	-28.63
N-Ethyl-o-crotonotoluidide	-39.81
NIALAMIDE	-63.84
NICORANDIL	-14.00
NICOTINAMIDE	-20.26
NICOTINE	2.90
Nicotinic Acid	-68.84
NIFEDIPINE	-241.22
NIFEKALANT HCl	-26.49
Nimetazepam	-18.85
NIMODIPINE	-150.38
NISOLDIPINE	-67.12
NITAZOXANIDE	-38.92
Nitrazepam	-43.67
NITRENDIPINE	-129.66
NITROFURANTOIN	-0.81
NIZATIDINE	-9.63
Nobiletin	-217.65
Norflex	-41.19
NORFLOXACIN	-30.60
Nornicotine	-5.64
Norpace	-58.43
Norpramin	-14.30
Novocain	-10.70
OFLOXACIN	37.67
OLANZAPINE	-39.83
OLIGOMYCIN C	-25.65
OLMESARTAN MEDOXOMIL	17.80
OLOPATADINE HCl	-4.64
OMEPRAZOLE	-74.37
Ondansetron	-44.81
ONDANSETRON HYDROCHLORIDE	-1.88
ORLISTAT	-26.07
ORMETOPRIM	-3.87
ORNIDAZOLE	-20.09
OXAPROZIN	-1.88
OXICONAZOLE NITRATE	-471.14
Oxiranecarboxylic acid, 2-[6-(4-chlorophenoxy)hexyl]-, ethyl ester- [CAS]	-119.90
OXYBUTYNIN CHLORIDE	7.90
Oxymetholone	37.00
OXYPHENONIUM BROMIDE	-0.88
Oxytetracycline hydrochloride	-32.15
ozagrel, monohydrochloride	-12.71
PALONOSETRON HCl	-50.02

Table A.1 Continued.

Pamelor	10.67
Pancuronium	-2.82
PANTOPRAZOLE SODIUM SALT	4.43
PARECOXIB Na	-41.08
Paroxetine	28.75
PAROXETINE	13.20
Paroxetine	29.11
PAZUFLOXACIN	-17.74
PD 81723	-101.66
PEFLOXACIN MESYLATE	2.49
Pemoline	-26.94
PENCICLOVIR	-9.46
Penicillin V	-29.64
Pentoxifylline	-6.30
PERGOLIDE MESYLATE	-34.66
PEROSPIRONE HCl	-221.05
PERPHENAZINE	4.55
Pfizerpen	-52.38
Phenelzine	0.69
PHENELZINE SULFATE SALT	-10.83
Phenergan	-60.34
PHENOTHIAZINE	-185.13
PHENPROBAMATE	-32.26
PHENTOLAMINE HCL	-25.56
Phylloquinone	16.86
Physostigmine	-34.58
PICEID	-48.05
Picrotin - Picrotoxinin	-7.74
PIDOTIMOD	-25.49
PILOCARPINE HYDROCHLORIDE	-24.04
Pinacidil monohydrate	-37.17
PINDOLOL	6.77
PIOGLITAZONE HCl	-37.91
PIPERACILLIN SODIUM SALT	-32.15
PIRENPERONE	-39.75
Piribedil	-17.22
Piroxicam	5.08
Pizotyline	-41.77
Podofilox	-74.63
Pramipexole	-17.68
PRAMIPEXOLE HCl	-10.50
PRAVASTATIN Sodium	-20.49
PRAZIQUANTEL	-32.70

Table A.1 Continued.

Prazosin	-74.72
Prednisolone	-71.01
PREDNISOLONE ACETATE	-58.18
Prednisone	-59.54
PRILOCAINE HYDROCHLORIDE	-56.61
Primaquine Diphosphate	-47.21
Primidone	-17.33
Priscoline	15.88
Pro-Amatine	-45.72
Pro-Banthine	-35.85
Probenecid	-25.57
PROCARBAZINE HYDROCHLORIDE	-10.24
PROGESTERONE	-135.52
Pronestyl	-33.25
Propofol	-96.14
Propylthiouracil	1.80
Prostaglandin E1	-281.47
Proxymetacaine	-66.72
Prozac	-0.35
PTEROSTILBENE	-111.36
Pyrazinamide	-28.09
Pyrazinecarboxamide, 3,5-diamino-N-(aminoiminomethyl)-6-chloro-[CAS]	-19.57
PYRIDINE-2-ALDOXIME METHOCHLORIDE	16.69
Pyrimethamine	4.91
QUETIAPINE HEMIFUMARATE	-55.53
Quinapril hydrochloride	-59.85
Quinidine hydrochloride monohydrate	-43.57
R(+)-SCH-23390 hydrochloride	-71.68
RABEPRAZOLE	-225.07
Raclopride	-38.11
RALTITREXED	6.53
RAMIPRIL	-24.47
RAMIPRIL	-27.13
Ranitidine hydrochloride	-7.39
Ranolazine dihydrochloride	-15.06
Reichsteins substanceÂ S	-24.58
REPAGLINIDE	24.07
Resveratrol	-80.06
Retinoic acid	-39.84
Ribavirin	-43.19
RIFABUTIN	-77.60
Rifabutin	-93.76
RIFAMPICIN	-13.15

Table A.1 Continued.

RIFAPENTINE	-64.54
RIFAPENTINE	-93.03
RIFAXIMIN	-35.08
Riluzole	-58.55
Rimcazole	-8.93
RISPERIDONE	-90.13
RITONAVIR	-145.33
RIZATRIPTAN BENZOATE	-35.64
ROFECOXIB	-19.89
ROLIPRAM	-197.91
ROLITETRACYCLINE	-89.46
ROPIVACAINE HCl	14.31
ROSIGLITAZONE HCl	-29.74
ROSIGLITAZONE MALEATE	-21.83
ROXATIDINE ACETATE HCl	-23.89
RU 24969	-100.81
RUFLOXACIN HCl	16.34
RUTIN	-7.79
Rythmol	-24.02
S(-)-Timolol maleate	-11.27
SALBUTAMOL SULFATE	-57.70
Salmeterol	-66.58
SAQUINAVIR MESYLATE	0.63
SB 205607	-87.65
SDM25N	-148.22
SECOISOLARICIRESINOL	7.48
Sertraline	30.75
Sertraline	6.39
Sibutramine	-74.85
SIBUTRAMINE HCl	-48.81
Simvastatin	-282.50
SKF 83566	85.21
Sonazine	-10.09
Sotalol hydrochloride	-14.64
SPECTINOMYCIN DIHYDROCHLORIDE PENTAHYDRATE	-80.03
Spironolactone	-89.91
SR 57,227A	4.17
Stanozolol	-168.50
STAVUDINE	-43.12
STAVUDINE	-36.06
Stiripentol	24.79
Sulfacetamide	-45.86
Sulfamethoxazole	-38.02
SULFASALAZINE	-7.99

Table A.1 Continued.

Sulfinpyrazone	-53.83
Sulfisoxazole	-37.08
Sulindac	-125.91
SUMATRIPTAN SUCCINATE	10.65
Symmetrel	-23.92
SYNEPHRINE	-12.39
TACROLIMUS	-176.39
TADALAFIL	28.66
Tamoxifen	-86.62
TAXIFOLIN-(+)	-15.27
TAXIFOLIN-(+/-)	-1.15
TEGASEROD MALEATE	21.65
TELITHROMYCIN	-73.24
TELMISARTAN	-84.77
TEMOZOLOMIDE	-3.15
Terazosin	-21.67
Terazosin	-8.54
TERBINAFINE HCl	-1025.30
Terbutaline Sulfate	-92.19
Testosterone	-79.38
Testosterone	-133.96
TETRACYCLINE	-58.86
TETRAETHYLTHIURAM DISULFIDE	-1275.81
TFMPP	16.24
Thalidomide	-28.80
Theophylline	-69.10
THIABENDAZOLE	-2.52
Thiophene, 5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-[CAS]	-50.82
THIORIDAZINE HYDROCHLORIDE	58.40
THIOTHIXENE	16.34
TIAGABINE HCl	-36.11
TIBOLONE	-131.53
TICLOPIDINE HCl	-242.87
TINIDAZOLE	-19.86
Tizanidine hydrochloride	-98.06
TOCAINIDE	-21.83
Tofranil	-286.92
TOLAZAMIDE	-44.51
Tolbutamide	39.25
TOLTERODINE TARTRATE	-27.08
TOPIRAMATE	-19.99
TOPOTECAN HCL	40.67
TORASEMIDE	-41.10

Table A.1 Continued.

TOREMIFENE CITRATE	-16.54
TOSUFLOXACIN TOSYLATE	-41.06
Tramadol	-25.90
TRANILAST	68.77
TRAZODONE HYDROCHLORIDE	-4.12
TREMULACIN	-315.25
TRIAMCINOLONE ACETONIDE	-22.19
Triamterene	1.47
TRICLABENDAZOLE	-155.68
Triclosan	-181.17
Trileptal	-10.76
TRIMEBUTINE MALEATE	-97.99
Trimethoprim	-0.16
TRIPELENNAMINE HYDROCHLORIDE	-34.08
Tripfluoperazine Hydrochloride	28.64
TRIPTOLIDE	-5.67
TROPICAMIDE	-60.08
TROPISETRON HCl	-6.79
TROXIPIDE	-24.60
TRYPTOLINE	-81.98
Tyzine	-35.39
URAPIDIL HYDROCHLORIDE	-26.34
Urecholine	-9.34
URSODEOXYCHOLIC ACID	-49.48
VALACICLOVIR HYDROCHLORIDE	-20.27
VALDECOXIB	-42.76
Valproic Acid	-98.54
VALSARTAN	-40.09
VARDENAFIL CITRATE	16.37
VECURONIUM BROMIDE	-25.16
VENLAFAXINE HCl	-36.29
VINCRISTINE SULFATE	-49.39
VINDESINE SULFATE	-61.35
VINORELBINE BITATRATE	-45.00
Viramune	-11.00
Vistaril Pamoate	17.41
VORICONAZOLE	-11.99
WARFARIN SODIUM	-12.62
Westcort	-49.55
XANTHINOL NICOTINATE	-56.27
Zacopride	-5.81
ZAFIRLUKAST	8.66
Zaleplon	33.51
ZERANOL	-87.34

Table A.1 Continued.

Zidovudine	-52.97
ZILEUTON	-85.00
Zolmitriptan	-30.51
ZOLPIDEM TARTRATE	-33.68
Zonisamide	-39.54

The NIH clinical collections I and II contain drug-like molecules with documented biological activity and a history of use in human clinical trials. These properties create a bias for biological activity that is non-specific with regard to AC2 inhibition. For example, SKF-83566 was validated as a direct small molecule AC2 inhibitor, but is also known to be a dopamine and serotonin receptor antagonist (see discussion in chapter 4). Therefore, it is desirable to identify chemical moieties and functional groups that confer specificity and/or potency for inhibition of AC2. As such, a modest structure-activity relationship study was designed by testing analogues of four compounds (SKF-83566, tranilast, loratadine, and oxymetholone, see Figure A.1 for chemical structures) that were identified and validated as modulators of AC2 activity (3671-5745, 1683-6987, 8005-4220, 4100-1558, and N050-006 were purchased from ChemDiv, San Diego, CA). All compounds were tested for the ability to modulate cAMP accumulation in response to activation of AC isoforms in HEK-hAC2 (activation by 50 nM PMA, 3 μM forskolin, and 300 nM PGE2), HEK-hAC1 (activation by 3 μM A23187), HEK-hAC5 (activation by 300 nM forskolin), and HEK-wt cells (activation by 3 μM forskolin) as described in chapter 4 (Table A.2). These experiments represent an initial small-scale structure-activity relationship study for the compounds that inhibit AC2 activity, while simultaneously counter-screening to examine the AC isoform selectivity of the compounds.

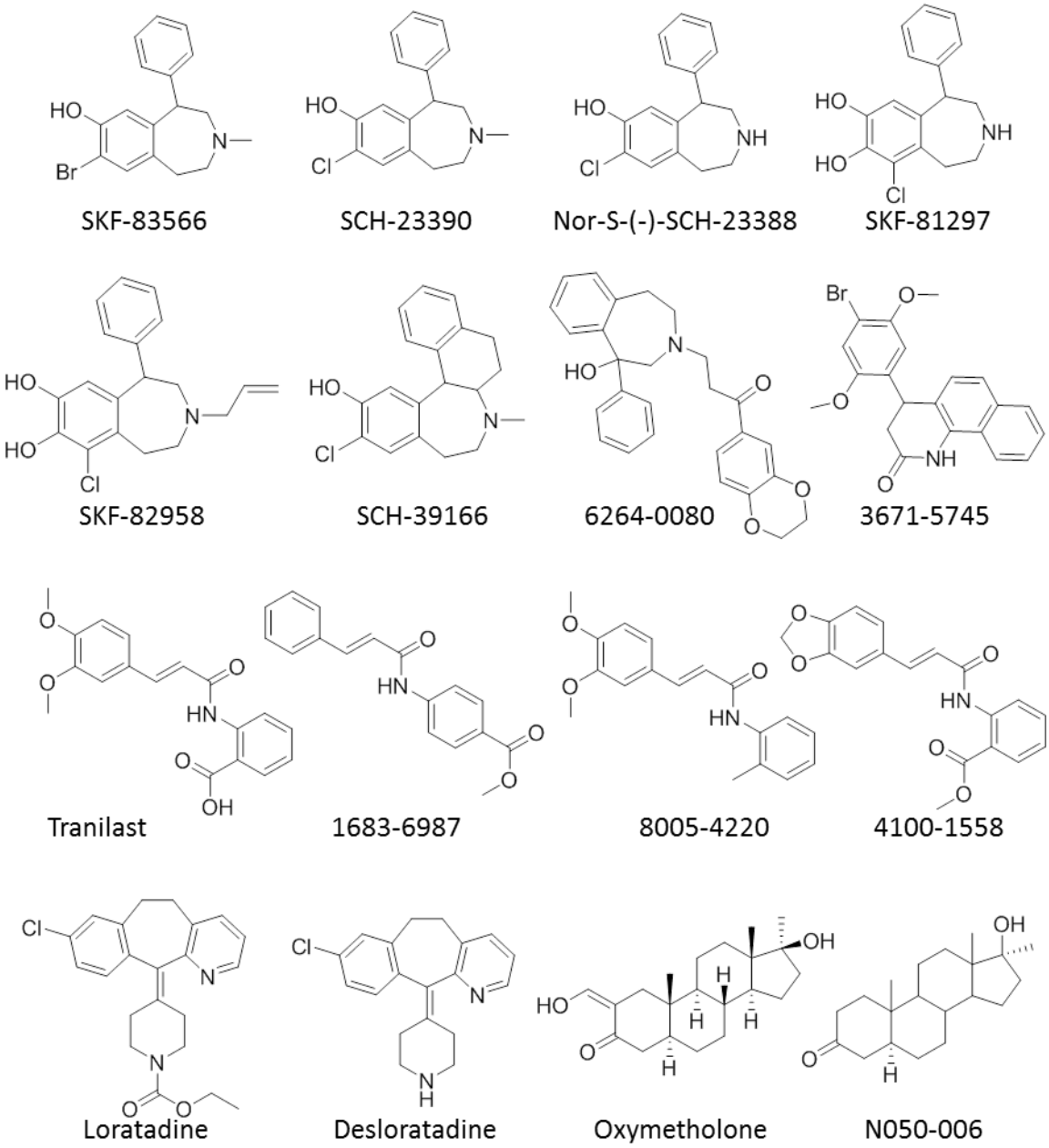

Figure A.1 Chemical structures of active compounds and their analogues.

Table A.2 AC isoform-selectivity profiles of test compounds in intact-cell studies. AC isoform selectivity was assessed by testing the ability of test compounds (30 µM) to modulate cAMP responses in HEK-hAC2 cells, HEK-hAC1 cells, HEK-hAC5 cells, and HEK-wt cells. Data are reported as a percent of the vehicle treatment condition and represent the Mean ± S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ compared to vehicle condition, one sample *t*-test compared to 100.

	AC2			**AC1**	**AC5**	**WT**
	50 nM PMA	**3 µM FSK**	**300 nM PGE2**	**3 µM A23187**	**300 nM FSK**	**3 µM FSK**
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
SKF-83566 HBr	16±0.66***	36±3.3**	20±2.6**	100±8.1	61±2.7**	85±3.0*
(+)-SCH23390 HCl	54±1.1***	74±1.7**	68±3.6*	120±6.6	74±5.7*	100±14
Nor-S-(-)-SCH 23388 HCl	77±7.9	71±4.1*	82±9.2	81±10	98±5.3	93±8.8
SKF81297 HBr	86±8.2	130±9.3	100±20	84±16	200±2.4***	260±8.4**
SKF82958 HBr	81±16	94±10	82±9	80±12	120±4.1	210±0.74***
SCH39166 HBr	98±11	100±1.5	110±8.9	ND	ND	110±14
6264-0080	310±36*	360±25**	360±75	33±4.4**	330±33*	240±19*
3671-5745	110±18	150±9.7*	130±6.4*	130±15	92±2.2	110±3.5
Oxymetholone	67±11	56±24	32±1.6***	75±9.7	100±9.7	84±11
N050-0006	89±12	110±5.7	110±7.6	100±15	99±2.9	100±4.4
Tranilast	33±5.0**	32±3.7**	55±2.8**	96±5.4	31±0.31***	60±6.4*
1683-6987	98±4.8	92±2.8	110±16	94±5.9	110±2.2*	130±5.6*
8005-4220	81±12	88±4.7	83±8.2	96±7.3	90±7.2	91±1.4*
4100-1558	88±7.9	110±1.8*	160±14	75±5.9	110±5.0	110±6.5
Loratadine	50±6.8*	21±1.3***	46±2.3**	120±7.8	16±0.80***	51±3.2**
Desloratadine	67±4.7*	110±3.5	85±5.8	78±7.5	84±4.0	89±1.9*

The development and initial success of a robust cell-based HTS assay for inhibitors of AC2 (see chapter 4) prompted further screening. In an effort to identify novel and diverse small molecule inhibitors of AC2, the Spectrum Collection (MicroSource Discovery Systems Inc.) was screened for inhibition of PMA-stimulated cAMP in HEK-hAC2 cells as described in chapter 4. The Spectrum Collection is a diverse chemical collection that contains 2,320 compounds (60% drugs, 25% natural products, and 15% other bioactive molecules) and provided the opportunity to extend the screening efforts initiated by screening the NIH clinical collections I and II. The complete results from the screening of the Spectrum Collection compounds (20 μM) for inhibition of 50 nM PMA-stimulated cAMP in HEK-hAC2 cells are reported in Table A.3.

Table A.3 Screening of the Spectrum Collection for inhibition of AC2 activity. The Spectrum Collection (20 µM) was screened for inhibition of PMA-stimulated (50 nM) cAMP accumulation in HEK-hAC2 cells using the Cisbio HTRF cAMP dynamic 2 detection methodology. The data represent the percent inhibition of the PMA-stimulated cAMP response in singlet.

Compound Name	**% Inhibition**
1-(2-METHOXYPHENYL)PIERAZINE HYDROCHLORIDE	-3.07
1,2alpha-EPOXYDEACETOXYDIHYDROGEDUNIN	-40.94
1,3,5-TRIMETHOXYBENZENE	-48.95
1,3-DIDEACETYL-7-DEACETOXY-7-OXOKHIVORIN	20.00
1,3-DIDEACETYLKHIVORIN	-0.27
1,4-NAPHTHOQUINONE	99.60
1,7-DIDEACETOXY-1,7-DIOXO-3-DEACETYLKHIVORIN	-25.67
10-HYDROXYCAMPTOTHECIN	37.10
11a-ACETOXYPROGESTERONE	-15.39
12a-HYDROXY-5-DEOXYDEHYDROMUNDUSERONE	-103.88
12a-HYDROXY-9-DEMETHYLMUNDUSERONE-8-CARBOXYLIC ACID	-1.59
13-METHYL-4,4-BISNOR-8,11,13-PODOCARPATRIEN-3-ONE	-89.76
14-METHOXY-4,4-BISNOR-8,11,13-PODOCARPATRIEN-3-ONE	-104.77
18alpha-GLYCYRRHETINIC ACID	-10.67
18-AMINOABIETA-8,11,13-TRIENE SULFATE	-21.67
1-HYDROXY-3,6,7-TRIMETHOXY-2,8-DIPRENYLXANTHONE	12.42
1-MONOPALMITIN	21.46
1-PHENYLBIGUANIDE HYDROCHLORIDE	1.78
1R,2S-PHENYLPROPYLAMINE	-37.14
2,2'-AZO-bis-2-AMINOPROPANE	5.68
2',2'-BISEPIGALLOCATECHIN DIGALLATE	18.01
2,3,4-TRIHYDROXY-4'-ETHOXYBENZOPHENONE	-16.64
2,3,4'-TRIHYDROXY-4-METHOXYBENZOPHENONE	12.05
2,3-DICHLORO-5,8-DIHYDROXYNAPTHOQUINONE	-75.35
2',3-DIHYDROXY-4,4',6'-TRIMETHOXYCHALCONE	-223.55
2,3-DIHYDROXY-4-METHOXY-4'-ETHOXYBENZOPHENONE	-65.85
2,3-DIHYDROXY-6,7-DICHLOROQUINOXALINE	-110.67
2,3-DIMERCAPTOSUCCINIC ACID	-92.54
2,4-DICHLOROPHENOXYACETIC ACID	19.65
2,4-DICHLOROPHENOXYBUTYRIC ACID	-90.34
2',4-DIHYDROXY-3,4',6'-TRIMETHOXYCHALCONE	-54.71
2',4'-DIHYDROXY-4-METHOXYCHALCONE	-28.22

Table A.3 Continued.

2',4'-DIHYDROXYCHALCONE	-121.12
2',4-DIHYDROXYCHALCONE	-121.33
2',4'-DIHYDROXYCHALCONE 4'-GLUCOSIDE	-29.60
2,4-DINITROPHENOL	-221.20
2',5'-DIHYDROXY-4-METHOXYCHALCONE	-355.99
2,5-DI-t-BUTYL-4-HYDROXYANISOLE	-753.17
2,6-DIHYDROXY-4-METHOXYTOLUENE	0.08
2,6-DIMETHOXYQUINONE	98.02
21-ACETOXYPREGNENOLONE	6.55
2-ACETYLPYRROLE	-14.10
2-AMINOBENZENESULFONAMIDE	-25.00
2-AMINOGUANIDINE HEMISULFATE	1.26
2-BENZOYL-5-METHOXYBENZOQUINONE	-42.64
2-HYDROXY-3,4-DIMETHOXYBENZOIC ACID	-11.99
2-HYDROXY-5 (6)EPOXY-TETRAHYDROCARYOPHYLLENE	-12.54
2-MERCAPTOBENZOTHIAZOLE	-138.94
2-METHOXY-5 (6)EPOXY-TETRAHYDROCARYOPHYLLENE	-10.31
2'-METHOXYFORMONETIN	8.29
2-METHOXYRESORCINOL	5.61
2-METHOXYXANTHONE	-150.57
2-METHYL GRAMINE	-45.27
2-METHYL-4-(PIPERIDIN-1-YLCARBOXY)-5-ISOPROPYLPHENYLTRIMETHYLAMMONIUM CHLORIDE	19.65
2-METHYL-5,7,8-TRIMETHOXYISOFLAVONE	29.32
2-METHYLENE-5-(2,5-DIOXOTETRAHYDROFURAN-3-YL)-6-OXO--10,10-DIMETHYLBICYCLO[7: 2: 0]UNDECANE	2.19
2-THIOURACIL	-8.15
3,16-DIDEOXYMEXICANOLIDE-3beta-DIOL	2.91
3,3'-DIINDOLYLMETHANE	-22.31
3,4',5,6,7-PENTAMETHOXYFLAVONE	-66.71
3,4-DIDESMETHYL-5-DESHYDROXY-3'-ETHOXYSCLEROIN	-1.99
3,4-DIMETHOXYCINNAMIC ACID	14.27
3,4-DIMETHOXYDALBERGIONE	88.27
3,4'-DIMETHOXYFLAVONE	-165.77
3,6-DIMETHOXYFLAVONE	-92.72
3,7-DIHYDROXYFLAVONE	22.49
3,7-DIMETHOXYFLAVONE	-75.57
3,7-EPOXYCARYOPHYLLAN-6-OL	1.78

Table A.3 Continued.

3,7-EPOXYCARYOPHYLLAN-6-ONE	-5.40
3-ACETAMIDOCOUMARIN	-35.65
3-ACETYLCOUMARIN	-138.94
3alpha-ACETOXYDIHYDRODEOXYGEDUNIN	-35.81
3alpha-HYDROXY-3-DEOXYANGOLENSIC ACID METHYL ESTER	1.07
3alpha-HYDROXY-4,4-BISNOR-8,11,13-PODOCARPATRIENE	10.19
3-AMINO-1,2,4-TRIAZOLE	-16.48
3-AMINO-beta-PINENE	-4.78
3beta-ACETOXYDEOXODIHYDROGEDUNIN	-10.45
3beta-HYDROXY-23,24-BISNORCHOL-5-ENIC ACID	-12.30
3beta-HYDROXYDEOXODIHYDRODEOXYGEDUNIN	-21.84
3beta-HYDROXYISOALLOSPIROST-9(11)-ENE	-6.63
3-DEACETYLKHIVORIN	-65.18
3-DEOXO-3beta-ACETOXYDEOXYDIHYDROGEDUNIN	-71.94
3-DEOXO-3beta-HYDROXYMEXICANOLIDE 16-ENOL ETHER	-13.90
3-DEOXY-3beta-HYDROXYANGOLENSIC ACID METHYL ESTER	-0.07
3-DESHYDROXYSAPPANOL TRIMETHYL ETHER	-36.43
3H-1,2-DITHIOLE-3-THIONE	-56.41
3-HYDROXY-3',4'-DIMETHOXYFLAVONE	-20.12
3-HYDROXY-4-(SUCCIN-2-YL)-CARYOLANE delta-LACTONE	-13.55
3-HYDROXYFLAVONE	-37.15
3-HYDROXYTYRAMINE	-12.69
3-ISOBUTYL-1-METHYLXANTHINE (IBMX)	-25.40
3-METHOXYCATECHOL	-46.70
3-METHYLORSELLINIC ACID	1.78
3-METHYLXANTHINE	6.78
3-NOR-3-OXOPANASINSAN-6-OL	4.61
3-OXOURSAN (28-13)OLIDE	-64.00
4-(3-BUTOXY-4-METHOXYBENZYL)IMIDAZOLIDIN-2-ONE	-171.13
4,4'-DIISOTHIOCYANOSTILBENE-2,2'-SUFONIC ACID SODIUM SALT	-45.60
4,4'-DIMETHOXYDALBERGIONE	-702.19
4-ACETOXYPHENOL	-1.05
4'-DEMETHYLEPIPODOPHYLLOTOXIN	-25.67
4-HYDROXY-6-METHYLPYRAN-2-ONE	-21.17
4-HYDROXYANTIPYRINE	-38.79
4'-HYDROXYCHALCONE	-32.24
4'-HYDROXYFLAVANONE	-59.61

Table A.3 Continued.

4-HYDROXYINDOLE	-25.58
4'-METHOXYCHALCONE	-100.19
4-METHOXYDALBERGIONE	-593.06
4-METHYLDAPHNETIN	-9.99
4-METHYLESCULETIN	-113.38
4-NAPHTHALIMIDOBUTYRIC ACID	16.66
4-NONYLPHENOL	-64.70
4-O-METHYLPHLORACETOPHENONE	-22.11
5,7-DIHYDROXY-4-METHYLCOUMARIN	34.78
5,7-DIHYDROXYISOFLAVONE	12.00
5alpha-ANDROSTAN-3,17-DIONE	-274.74
5alpha-CHOLESTAN-3beta-OL-6-ONE	26.26
5alpha-CHOLESTANOL	-14.02
5-AMINOPENTANOIC ACID HYDROCHLORIDE	-11.53
5-CHLOROINDOLE-2-CARBOXYLIC ACID	-2.24
5-FLUOROINDOLE-2-CARBOXYLIC ACID	2.62
5-HYDROXY-2',4',7,8-TETRAMETHOXYFLAVONE	-86.69
5-METHYLHYDANTOIN	-9.77
6,2'-DIMETHOXYFLAVONE	-68.45
6,3'-DIMETHOXYFLAVONE	-17.28
6,4'-DIHYDROXYFLAVONE	-10.37
6,7-DICHLORO-3-HYDROXY-2-QUINOXALINECARBOXYLIC ACID	24.66
6alpha-METHYLPREDNISOLONE ACETATE	-12.77
6-AMINONICOTINAMIDE	-2.19
6-HYDROXYANGOLENSIC ACID METHYL ESTER	0.47
6-HYDROXYFLAVONE	-61.41
6-HYDROXYTROPINONE	-26.64
7,2'-DIHYDROXYFLAVONE	14.40
7,3'-DIMETHOXYFLAVONE	-49.76
7,4'-DIHYDROXYFLAVONE	15.46
7,4'-DIMETHOXYISOFLAVONE	-2.81
7,8-DIHYDROXYFLAVONE	-133.38
7-AMINOCEPHALOSPORANIC ACID	-32.45
7-DEACETOXY-7-OXOKHIVORIN	-68.27
7-DEACETYLKHIVORIN	8.93
7-DESACETOXY-6,7-DEHYDROGEDUNIN	10.81
7-DESHYDROXYPYROGALLIN-4-CARBOXYLIC ACID	-8.07

Table A.3 Continued.

7-HYDROXYETHYLTHEOPHYLLINE	-14.68
7-HYDROXYFLAVONE	5.74
7-NITROINDAZOLE	-45.27
7-OXOCHOLESTEROL	-50.80
8beta-HYDROXYCARAPIN, 3,8-HEMIACETAL	-36.95
8-CYCLOPENTYLTHEOPHYLLINE	-12.07
8-HYDROXYCARAPINIC ACID	-1.19
ABACAVIR SULFATE	-13.47
ABAMECTIN (avermectin B1a shown)	-46.12
ABIENOL	-307.12
ABIETIC ACID	-140.80
ACACETIN DIACETATE	3.52
ACADESINE	0.52
ACAMPROSATE CALCIUM	10.25
ACARBOSE	-21.61
ACEBUTOLOL HYDROCHLORIDE	-12.92
ACECAINIDE HYDROCHLORIDE	-13.25
ACECLIDINE	-10.58
ACEDAPSONE	-23.80
ACEDOBEN	-20.58
ACEGLUTAMIDE	-2.60
ACEMETACIN	-18.04
ACENOCOUMAROL	-13.08
ACEPROMAZINE MALEATE	41.78
ACESULFAME POTASSIUM	-9.28
ACETAMINOPHEN	-6.04
ACETAMINOSALOL	23.48
ACETANILIDE	8.83
ACETARSOL	-11.61
ACETAZOLAMIDE	13.90
ACETOHEXAMIDE	2.68
ACETOHYDROXAMIC ACID	17.87
ACETOPHENAZINE MALEATE	7.80
ACETOSYRINGONE	-26.37
ACETRIAZOIC ACID	14.96
ACETYL ISOGAMBOGIC ACID	-31.10
ACETYLCARNITINE	-33.98

Table A.3 Continued.

ACETYLCHOLINE CHLORIDE	13.15
ACETYLCYSTEINE	-0.18
ACETYLGLUCOSAMINE	-56.03
ACETYL-L-LEUCINE	-12.74
ACETYLPHENYLALANINE	-29.13
ACEXAMIC ACID	-25.66
ACIPIMOX	5.78
ACONITIC ACID	0.74
ACONITINE	-0.62
ACRIFLAVINIUM HYDROCHLORIDE	60.54
ACRISORCIN	-19.99
ACTINONIN	-43.23
ACYCLOVIR	-3.55
ADENINE	4.95
ADENOSINE	-143.95
ADENOSINE PHOSPHATE	-35.03
ADIPHENINE HYDROCHLORIDE	-30.87
ADONITOL	-13.78
ADRENOLONE HYDROCHLORIDE	-35.34
AESCULIN	31.99
AGARIC ACID	5.80
AGELASINE (stereochemistry of diterpene unknown)	-21.88
AGMATINE SULFATE	10.86
AJMALINE	1.31
AKLOMIDE	0.32
ALANYL-dl-LEUCINE	10.87
ALAPROCLATE	4.93
ALBENDAZOLE	-23.68
ALBUTEROL	-29.04
ALCLOMETAZONE DIPROPIONATE	1.24
ALENDRONATE SODIUM	-25.22
ALEURETIC ACID	25.65
ALEXIDINE HYDROCHLORIDE	-17.11
ALFLUZOSIN	7.19
ALGESTONE ACETOPHENIDE	-116.80
ALISKIREN HEMIFUMARATE	14.57
ALIZARIN	-48.10

Table A.3 Continued.

ALLANTOIN	-10.14
ALLOPURINOL	-26.56
ALLOXAN	-38.79
ALLYLISOTHIOCYANATE	-40.59
ALMOTRIPTAN	-26.32
alpha-CYANO-3-HYDROXYCINNAMIC ACID	-12.69
alpha-CYANO-4-HYDROXYCINNAMIC ACID	-22.14
alpha-DIHYDROGEDUNOL	-44.72
alpha-HYDROXYDEOXYCHOLIC ACID	-25.00
alpha-MANGOSTIN	-78.42
alpha-METHYL-L-TYROSINE	-1.26
alpha-TOCHOPHEROL	-2.62
alpha-TOCHOPHERYL ACETATE	-9.96
alpha-TOXICAROL (dl)	-84.54
ALPINETIN METHYL ETHER	-9.99
ALPRENOLOL	4.99
ALRESTATIN	-5.76
ALTHIAZIDE	-18.17
ALTRENOGEST	-37.75
ALTRETAMINE	-46.56
ALVERINE CITRATE	-12.25
AMANTADINE HYDROCHLORIDE	-29.46
AMBROXOL HYDROCHLORIDE	18.10
AMCINONIDE	5.86
AMIFOSTINE	-28.05
AMIKACIN SULFATE	-20.17
AMILORIDE HYDROCHLORIDE	5.53
AMINACRINE	-20.42
AMINOBENZTROPINE	-7.07
AMINOCAPROIC ACID	-7.59
AMINOCYCLOPROPANECARBOXYLIC ACID	-3.20
AMINOETHOXYDIPHENYLBORANE	-245.61
AMINOETHYLISOTHIOUREA DIHYDROBROMIDE	-2.24
AMINOGLUTETHIMIDE	-4.43
AMINOHIPPURIC ACID	-14.06
AMINOHYDROXYBUTYRIC ACID	-11.85
AMINOLEVULINIC ACID HYDROCHLORIDE	-5.04

Table A.3 Continued.

AMINOPENTAMIDE SULFATE	-11.35
AMINOPTERIN	12.62
AMINOPYRINE	9.78
AMINOSALICYLATE SODIUM	-13.99
AMINOTHIAZOLE	23.05
AMIODARONE HYDROCHLORIDE	-258.09
AMIPRILOSE	-40.59
AMISULPRIDE	-12.25
AMITRAZ	-50.85
AMITRIPTYLINE HYDROCHLORIDE	-8.22
AMLODIPINE BESYLATE	56.23
AMMONIUM LACTATE	-7.86
AMODIAQUINE DIHYDROCHLORIDE	-23.71
AMOXAPINE	7.07
AMOXICILLIN	13.90
AMPHOTERICIN B	-8.29
AMPICILLIN SODIUM	15.31
AMPIROXICAM	-32.62
AMPROLIUM	-27.90
AMPYRONE	3.30
AMPYZINE SULFATE	9.37
AMSACRINE	-20.19
AMYGDALIN	-0.92
ANABASAMINE HYDROCHLORIDE	-10.67
ANABASINE HYDROCHLORIDE	-0.85
ANAGRELIDE HYDROCHLORIDE	-78.00
ANASTROZOLE	2.40
ANCITABINE HYDROCHLORIDE	-12.50
ANDROSTA-1,4-DIEN-3,17-DIONE	-60.76
ANDROSTERONE	-196.30
ANDROSTERONE ACETATE	-371.76
ANEBROMPHENIRAMINE MALEATE	20.61
ANETHOLE	-45.28
ANGOLENSIN (R)	-64.73
ANHYDROBRAZILIC ACID	-16.16
ANIRACETAM	-4.05
ANISINDIONE	7.56

Table A.3 Continued.

ANISODAMINE HYDROBROMIDE	6.34
ANISOMYCIN	-24.59
ANTAZOLINE PHOSPHATE	1.66
ANTHOTHECOL	-133.15
ANTHRALIN	-46.12
ANTHRAQUINONE	-63.47
ANTIAROL	56.56
ANTIMYCIN A (A1 shown)	-185.07
ANTIPYRINE	12.11
APHYLLIC ACID	-42.44
APIGENIN	-17.04
APIGENIN DIMETHYL ETHER	-25.04
APIIN	-22.50
APIOLE	-174.46
APOMORPHINE HYDROCHLORIDE	0.19
APOTOXICAROL	-82.97
APRAMYCIN	13.86
ARABITOL(D)	-3.54
ARBUTIN	-3.75
ARCAINE SULFATE	0.67
ARECOLINE HYDROBROMIDE	8.04
ARGININE HYDROCHLORIDE	-24.91
ARIPIPRAZOLE	-225.19
ARSANILIC ACID	-16.53
ARSENIC TRIOXIDE	-10.41
ARTEMETHER	26.30
ARTEMISIN	-2.88
ARTEMISININ	-57.80
ARTENIMOL	-5.09
ARTESUNATE	-11.91
ARTHONIOIC ACID	-123.25
ASARYLALDEHYDE	2.59
ASCORBIC ACID	40.72
ASCORBYL PALMITATE	22.67
ASIATIC ACID	-12.20
ASPARTAME	20.78
ASPIRIN	22.77

Table A.3 Continued.

ASTAXANTHIN	-26.21
ASTEMIZOLE	43.15
ASTRAGALOSIDE IV	-21.84
ATENOLOL	-5.65
ATOMOXETINE HYDROCHLORIDE	-13.55
ATORVASTATIN CALCIUM	-8.22
ATOVAQUONE	5.03
ATRACURIUM BESYLATE	4.08
ATRANORIN	-110.98
ATROPINE SULFATE	-19.17
AURAPTENE	-6.43
AURIN TRICARBOXYLIC ACID	-5.26
AUROTHIOGLUCOSE	-11.94
AVERMECTIN A1a	-72.25
AVOBENZONE	-404.39
AVOCADANOFURAN	-14.86
AVOCADENOFURAN	-4.02
AVOCADYNE	38.05
AVOCADYNE ACETATE	25.46
AVOCADYNOFURAN	-3.41
AVOCATIN A	50.49
AVOCATIN B	5.05
AZACITIDINE	5.90
AZADIRACHTIN	-23.88
AZAPERONE	-44.02
AZASERINE	-36.60
AZATADINE MALEATE	-19.21
AZATHIOPRINE	-27.69
AZELAIC ACID	22.19
AZELASTINE HYDROCHLORIDE	13.97
AZITHROMYCIN	-0.08
AZLOCILLIN SODIUM	-23.14
AZOBENZENE	-61.67
AZTREONAM	23.85
BACAMPICILLIN HYDROCHLORIDE	-16.95
BACCATIN III	5.99
BACITRACIN	6.12

Table A.3 Continued.

BACLOFEN	-6.73
BAICALEIN	-60.64
BAICALIN	6.30
BALSALAZIDE DISODIUM	17.38
BAMBUTEROL HYDROCHLORIDE	1.31
BARBITAL	-20.14
BATYL ALCOHOL	0.13
BECLAMIDE	-1.14
BECLOMETHASONE DIPROPIONATE	-36.00
BEKANAMYCIN SULFATE	10.76
BEMOTRIZINOL	-6.10
BENAZEPRIL HYDROCHLORIDE	26.21
BENDROFLUMETHIAZIDE	24.53
BENFLUOREX HYDROCHLORIDE	-19.43
BENFOTIAMINE	11.77
BENOXINATE HYDROCHLORIDE	3.39
BENSERAZIDE HYDROCHLORIDE	-41.68
BENURESTAT	12.69
BENZALKONIUM CHLORIDE	-6.49
BENZANTHRONE	-1067.13
BENZBROMARONE	-115.15
BENZETHONIUM CHLORIDE	-43.26
BENZO[a]PYRENE	-108.23
BENZOCAINE	21.47
BENZOIC ACID	-23.04
BENZONATATE	-37.01
BENZOXIQUINE	3.07
BENZOYL PEROXIDE	-17.87
BENZOYLPAS	5.74
BENZTHIAZIDE	7.15
BENZYDAMINE HYDROCHLORIDE	-14.24
BENZYL ALCOHOL	-24.54
BENZYL BENZOATE	-16.03
BENZYL ISOTHIOCYANATE	-22.37
BEPHENIUM HYDROXYNAPTHOATE	13.33
BEPRIDIL HYDROCHLORIDE	-46.09
BERBAMINE HYDROCHLORIDE	-9.92

Table A.3 Continued.

BERBERINE CHLORIDE	2.95
BERGAPTEN	-32.78
BERGENIN	7.56
beta-AMYRIN ACETATE	6.85
beta-CAROTENE	-111.70
beta-CARYOPHYLLENE ALCOHOL	-6.45
beta-ESCIN	41.97
BETAHISTINE HYDROCHLORIDE	-14.46
BETAINE HYDROCHLORIDE	-10.27
BETAMETHASONE	-20.63
BETAMETHASONE 17,21-DIPROPIONATE	13.86
BETAMETHASONE ACETATE	-1.80
BETAMETHASONE SODIUM PHOSPHATE	7.35
BETAMETHASONE VALERATE	6.83
BETAMIPRON	-8.68
beta-NAPHTHOL	-256.18
beta-PELTATIN	-11.36
beta-SITOSTEROL	-13.59
beta-TOXICAROL	-146.05
BETAXALOL HYDROCHLORIDE	7.13
BETAZOLE HYDROCHLORIDE	5.83
BETHANECHOL CHLORIDE	-7.67
BETULIN	17.44
BETULINIC ACID	-148.36
BEZAFIBRATE	1.04
BICALUTAMIDE	-40.83
BICUCULLINE (+)	-12.12
BICUCULLINE(-) METHIODIDE	-35.95
BIFONAZOLE	-58.83
BILIRUBIN	-68.13
BIOCHANIN A	-46.37
BIOTIN	-6.56
BIPERIDEN	-7.53
BISACODYL	-146.21
BISANHYDRORUTILANTINONE	-620.91
BISMUTH SUBSALICYLATE	-43.00
BISOCTRIZOLE	-56.42

Table A.3 Continued.

BISOPROLOL FUMARATE	10.60
BISPHENOL A	-58.69
BISSALICYL FUMARATE	1.75
BITHIONATE SODIUM	-139.66
BITOSCANATE	11.25
BIXIN	-23.21
BLASTICIDIN S	19.37
BLEOMYCIN (bleomycin B2 shown)	-0.11
BOLDINE	10.19
BORNEOL	-1.14
BORNYL ACETATE	-28.15
BOVINOCIDIN (3-nitropropionic acid)	31.38
BRAZILEIN	13.55
BRAZILIN	17.12
BRINZOLAMIDE	-2.09
BROMHEXINE HYDROCHLORIDE	-452.06
BROMINDIONE	4.74
BROMO-3-HYDROXY-4-(SUCCIN-2-YL)-CARYOLANE gamma-LACTONE	-0.18
BROMOCRIPTINE MESYLATE	-38.09
BROMOPRIDE	-10.14
BROMPERIDOL	40.08
BRUCINE	-11.18
BUCETIN	21.31
BUCLADESINE	-2405.69
BUDESONIDE	-16.64
BUFEXAMAC	-118.56
BUFLOMEDIL HYDROCHLORIDE	7.20
BUMETANIDE	17.98
BUPIVACAINE HYDROCHLORIDE	-27.00
BUPROPION	-25.84
BUSPIRONE HYDROCHLORIDE	5.62
BUSSEIN	-37.73
BUSULFAN	-9.96
BUTACAINE	13.42
BUTAMBEN	-37.52
BUTOCONAZOLE	-44.49
BUTYL PARABEN	-82.45

Table A.3 Continued.

BUTYLATED HYDROXYTOLUENE	-450.16
CACODYLIC ACID	-39.64
CADAVERINE TARTRATE	13.03
CADIN-4-EN-10-OL	-29.23
CAFESTOL	11.76
CAFESTOL ACETATE	-12.20
CAFFEIC ACID	-1.19
CAFFEINE	13.32
CALCEIN	24.19
CALCIUM GLUCEPTATE	13.61
CAMPHOR (1R)	21.52
CAMPTOTHECIN	-12.33
CAMYLOFINE DIHYDROCHLORIDE	-102.32
CANAVANINE	-2.04
CANDESARTAN	-1.28
CANDICIDIN	-163.40
CANRENOIC ACID, POTASSIUM SALT	18.36
CANRENONE	-34.75
CANTHARIDIN	-13.98
CANTHAXANTHIN (euglenanone)	-7.31
CAPECITABINE	-5.88
CAPOBENIC ACID	-6.49
CAPREOMYCIN SULFATE	-15.75
CAPSAICIN	-18.80
CAPSANTHIN	-79.48
CAPTAMINE	-12.24
CAPTAN	99.01
CAPTOPRIL	-36.27
CARAPIN	-6.28
CARAPIN-8(9)-ENE	-19.50
CARBACHOL	-13.81
CARBADOX	14.48
CARBAMAZEPINE	7.48
CARBARSONE	-18.61
CARBENICILLIN DISODIUM	11.39
CARBENOXOLONE SODIUM	-15.48
CARBETAPENTANE CITRATE	-120.51

Table A.3 Continued.

CARBIDOPA	-48.43
CARBINOXAMINE MALEATE	-9.72
CARBOPLATIN	-27.67
CARISOPRODOL	-17.63
CARMINIC ACID	17.85
CARMOFUR	12.06
CARMUSTINE	-76.58
CARNITINE (dl) HYDROCHLORIDE	8.23
CARNOSIC ACID	-32.45
CARNOSINE	-33.66
CARPROFEN	-23.52
CARSALAM	-29.51
CARTEOLOL HYDROCHLORIDE	10.14
CARVEDILOL	-75.51
CARVEDILOL PHOSPHATE	-177.70
CARYLOPHYLLENE OXIDE	-53.97
CARYOPHYLLENE [t(-)]	-188.99
CARZENIDE	7.68
CASANTHRANOL [cascaroside A shown]	-69.10
CATECHIN PENTAACETATE	-139.68
CATECHIN TETRAMETHYLETHER	-50.60
CEAROIN	-60.02
CEDRELONE	-258.13
CEDROL	-107.01
CEDRYL ACETATE	-220.43
CEFACLOR	-3.79
CEFADROXIL	-1.87
CEFALONIUM	18.77
CEFAMANDOLE NAFATE	-13.64
CEFAMANDOLE SODIUM	1.14
CEFAZOLIN SODIUM	18.91
CEFDINIR	-1.32
CEFDITORIN PIVOXIL	-48.78
CEFEPIME HYDROCHLORIDE	5.72
CEFMENOXIME HYDROCHLORIDE	-13.20
CEFMETAZOLE SODIUM	-2.44
CEFONICID SODIUM	4.61

Table A.3 Continued.

CEFOPERAZONE	-2.69
CEFORANIDE	-4.36
CEFOTAXIME SODIUM	-14.87
CEFOTETAN	17.04
CEFOXITIN SODIUM	-18.35
CEFPIRAMIDE	-5.52
CEFPODOXIME PROXETIL	-22.23
CEFPROZIL	3.96
CEFSULODIN SODIUM	-9.96
CEFTAZIDIME	3.26
CEFTIBUTEN	-3.20
CEFTIOFUR HYDROCHLORIDE	-7.64
CEFTRIAXONE SODIUM TRIHYDRATE	-0.28
CEFUROXIME AXETIL	9.24
CEFUROXIME SODIUM	-5.01
CELASTROL	-307.12
CELECOXIB	16.46
CELLOBIOSE (D[+])	12.87
CEPHALEXIN	21.42
CEPHALOSPORIN C SODIUM	18.15
CEPHALOTHIN SODIUM	-1.73
CEPHAPIRIN SODIUM	18.42
CEPHARANTHINE	-3.38
CEPHRADINE	6.90
CETIRIZINE HYDROCHLORIDE	2.30
CETRIMONIUM BROMIDE	29.30
CETYLPYRIDINIUM CHLORIDE	-8.92
CHAULMOOGRIC ACID	-23.21
CHENODIOL	-13.88
CHICAGO SKY BLUE	109.19
CHINIOFON	12.73
CHLORALOSE	-3.73
CHLORAMBUCIL	-10.27
CHLORAMINE-T	15.88
CHLORAMPHENICOL	22.42
CHLORAMPHENICOL PALMITATE	5.59
CHLORAMPHENICOL SODIUM SUCCINATE	-4.35

Table A.3 Continued.

CHLORANIL	81.80
CHLORCYCLIZINE HYDROCHLORIDE	-26.97
CHLORHEXIDINE HYDROCHLORIDE	-67.47
CHLORINDIONE	6.83
CHLORMADINONE ACETATE	-1.87
CHLORMEZANONE	5.90
CHLOROCRESOL	-49.66
CHLOROGUANIDE HYDROCHLORIDE	-0.32
CHLOROPHYLLIDE Cu COMPLEX Na SALT	70.63
CHLOROPYRAMINE HYDROCHLORIDE	-3.88
CHLOROQUINE DIPHOSPHATE	-0.03
CHLOROTHIAZIDE	18.85
CHLOROXINE	-46.56
CHLOROXYLENOL	-81.46
CHLORPHENIRAMINE (S) MALEATE	-6.70
CHLORPROMAZINE	25.27
CHLORPROPAMIDE	-7.29
CHLORPROTHIXENE HYDROCHLORIDE	50.50
CHLORPYRIFOS	-232.94
CHLORQUINALDOL	19.54
CHLORTETRACYCLINE HYDROCHLORIDE	-27.53
CHLORTHALIDONE	-31.98
CHLORZOXAZONE	-25.02
CHOL-11-ENIC ACID	-16.10
CHOLECALCIFEROL	-100.33
CHOLEST-4,6-DIEN-3-ONE	-19.32
CHOLEST-5-EN-3-ONE	-15.55
CHOLESTAN-3-ONE	-18.91
CHOLESTANE	18.21
CHOLESTEROL	-6.70
CHOLESTERYL ACETATE	-15.00
CHOLIC ACID	-33.87
CHOLIC ACID, METHYL ESTER	-60.12
CHOLINE CHLORIDE	-4.51
CHROMOCARB	-10.99
CHRYSANTHEMIC ACID	-23.61
CHRYSANTHEMIC ACID, ETHYL ESTER	-3.13

Table A.3 Continued.

CHRYSANTHEMYL ALCOHOL	-21.70
CHRYSAROBIN	-535.96
CHRYSIN	-69.57
CHRYSIN DIMETHYL ETHER	-54.22
CHRYSOPHANOL	-300.27
CHUKRASIN METHYL ETHER	-25.67
CIANIDANOL	-12.01
CICLOPIROX OLAMINE	-56.28
CILOSTAZOL	-5.26
CIMETIDINE	19.96
CINCHONIDINE	-11.09
CINCHONINE	-5.08
CINCHOPHEN	-1.07
CINNARAZINE	-449.99
CINOXACIN	-30.08
CINTRIAMIDE	3.96
CIPROFIBRATE	22.54
CIPROFLOXACIN	-12.92
CISAPRIDE	-72.57
CISPLATIN	-57.00
CITALOPRAM HYDROBROMIDE	11.23
CITICOLINE	0.25
CITIOLONE	11.55
CITRININ	-39.10
CITROPTEN	12.64
CITRULLINE	-10.67
CLARITHROMYCIN	-12.60
CLAVULANATE LITHIUM	-4.61
CLEMASTINE FUMARATE	12.35
CLEMIZOLE HYDROCHLORIDE	-14.62
CLENBUTEROL HYDROCHLORIDE	-27.86
CLIDINIUM BROMIDE	2.73
CLIMBAZOLE	11.60
CLINAFOXACIN HYDROCHLORIDE	9.25
CLINDAMYCIN HYDROCHLORIDE	7.25
CLINDAMYCIN PALMITATE HYDROCHLORIDE	-17.62
CLIOQUINOL	-4.00

Table A.3 Continued.

CLOBETASOL PROPIONATE	11.91
CLOFARABINE	-56.99
CLOFAZIMINE	-55.17
CLOFIBRATE	-1.59
CLOFIBRIC ACID	-6.87
CLOFILIUM TOSYLATE	-1.32
CLOFOCTOL	-439.91
CLOMIPHENE CITRATE	1.75
CLOMIPRAMINE HYDROCHLORIDE	-58.86
CLONAZEPAM	-13.66
CLONIDINE HYDROCHLORIDE	-33.09
CLOPERASTINE HYDROCHLORIDE	-1.76
CLOPIDOGREL SULFATE	1.77
CLOPIDOL	-11.58
CLORGILINE HYDROCHLORIDE	-1.21
CLORSULON	15.34
CLOSANTEL	0.82
CLOTRIMAZOLE	-80.48
CLOVANEDIOL DIACETATE	-62.30
CLOXACILLIN SODIUM	6.76
CLOXYQUIN	-104.05
CLOZAPINE	35.04
COLCHICINE	14.14
COLESEVALAM HYDROCHLORIDE (high mol wt copolymer @10mg/ml)	-11.24
COLFORSIN	-3268.26
COLISTIMETHATE SODIUM	34.85
COLISTIN SULFATE	-35.98
CONESSINE	12.20
CONVALLATOXIN	-10.67
CORALYNE CHLORIDE	6.85
CORTISONE	-28.25
CORTISONE ACETATE	-33.50
CORYNANTHINE	-18.21
COTARNINE CHLORIDE	-7.90
COTININE	-3.22
COUMARIN	-57.97
COUMOPHOS	0.65

Table A.3 Continued.

CREATININE	12.64
CRESOL	-19.72
CRESOPIRINE	-11.99
CROMOLYN SODIUM	0.65
CROTAMITON	-22.77
CRUSTECDYSONE	-13.13
CRYOFLURANE	-26.52
CRYPTOTANSHINONE	-630.03
CURCUMIN	-484.54
CYANOCOBALAMIN	3.11
CYCLAMIC ACID	9.58
CYCLANDELATE	-81.68
CYCLIZINE	-2.93
CYCLOBENZAPRINE HYDROCHLORIDE	17.10
CYCLOCREATINE	-0.18
CYCLOHEXIMIDE	8.53
CYCLOLEUCINE	10.02
CYCLOPENTOLATE HYDROCHLORIDE	-26.56
CYCLOPHOSPHAMIDE	-3.93
CYCLOSERINE (D)	-7.88
CYCLOSPORINE	-2.36
CYCLOTHIAZIDE	4.54
CYCLOVERATRYLENE	-9.84
CYPERMETHRIN	-144.72
CYPROHEPTADINE HYDROCHLORIDE	-22.21
CYPROTERONE	-1.28
CYPROTERONE ACETATE	6.58
CYROMAZINE	-0.72
CYSTAMINE DIHYDROCHLORIDE	-16.96
CYSTEAMINE HYDROCHLORIDE	3.84
CYSTEINE HYDROCHLORIDE	19.12
CYSTINE	-1.97
CYTARABINE	2.86
CYTIDINE	7.99
CYTISINE	3.28
d,l-threo-3-HYDROXYASPARTIC ACID	-7.53
DACARBAZINE	-25.18

Table A.3 Continued.

DACTINOMYCIN	-25.95
DALBERGIONE	12.50
DALBERGIONE, 4-METHOXY-4'-HYDROXY-	-30.25
DANAZOL	-30.48
DANTHRON	-250.19
DANTROLENE SODIUM	19.91
DAPSONE	2.92
DARIFENACIN HYDROBROMIDE	-38.06
DASATINIB	-12.94
DAUNORUBICIN	10.88
DEACETOXY(7)-7-OXOKHIVORINIC ACID	-4.39
DEACETOXY-7-OXOGEDUNIN	-92.69
DEACETYLGEDUNIN	-23.26
DEBRISOQUIN SULFATE	-27.48
DECAHYDROGAMBOGIC ACID	-445.84
DECAMETHONIUM BROMIDE	-23.75
DECOQUINATE	-24.58
DEFERIPRONE	-28.25
DEFEROXAMINE MESYLATE	8.60
DEGUELIN(-)	-92.20
DEHYDROABIETAMIDE	-48.61
DEHYDROACETIC ACID	25.15
DEHYDROCHOLATE SODIUM	-6.11
DEHYDROCHOLIC ACID	8.23
DEHYDRODIHYDROROTENONE	-13.55
DEHYDROROTENONE	-15.76
DEHYDROVARIABILIN	-287.74
DEMECLOCYCLINE HYDROCHLORIDE	-8.93
DEMETHYLNOBILETIN	-150.16
DENATONIUM BENZOATE	-9.12
DEOXYADENOSINE	68.04
DEOXYANDIROBIN	-10.99
DEOXYCHOLIC ACID	-4.59
DEOXYGEDUNIN	-50.92
DEOXYKHIVORIN	-8.72
DEOXYSAPPANONE B 7,3'-DIMETHYL ETHER	-116.45
DEOXYSAPPANONE B 7,3'-DIMETHYL ETHER ACETATE	-43.29

Table A.3 Continued.

DEOXYSAPPANONE B 7,4'-DIMETHYL ETHER	-128.48
DEOXYSAPPANONE B TRIMETHYL ETHER	-113.13
DEQUALINIUM CHLORIDE	-14.70
DERACOXIB	-20.01
DERRUSNIN	-28.30
DERRUSTONE	38.95
DESACETYL (7)KHIVORINIC ACID, METHYL ESTER	-7.23
DESACETYLCOLFORSIN	-689.41
DESIPRAMINE HYDROCHLORIDE	1.54
DESLORATADINE HYDROCHLORIDE	-72.14
DESLORATIDINE	35.35
DESONIDE	-9.95
DESOXYCORTICOSTERONE ACETATE	-17.81
DESOXYMETASONE	-2.50
DESOXYPEGANINE HYDROCHLORIDE	-5.26
DESVENLAFAXINE SUCCINATE	-41.55
DEXAMETHASONE	33.87
DEXAMETHASONE ACETATE	12.64
DEXAMETHASONE SODIUM PHOSPHATE	-1.16
DEXCHLORPHENIRAMINE MALEATE	-12.52
DEXIBUPROFEN	-36.90
DEXLANSOPRAZOLE	-19.53
DEXPANTHENOL	0.90
DEXPROPRANOLOL HYDROCHLORIDE	23.31
DIACERIN	-10.83
DIACETAMATE	-45.41
DIALLYL SULFIDE	-19.50
DIATRIZOIC ACID	6.70
DIAVERIDINE	-52.63
DIAZOXIDE	17.41
DIBEKACIN	-34.02
DIBENZOTHIOPHENE	-202.58
DIBENZOYLMETHANE	-63.72
DIBUCAINE HYDROCHLORIDE	-25.02
DIBUTYL PHTHALATE	-46.34
DICHLORISONE ACETATE	-10.99
DICHLORVOS	-26.61

Table A.3 Continued.

DICLAZURIL	-10.33
DICLOFENAC SODIUM	-37.83
DICLOXACILLIN SODIUM	-7.81
DICTAMNINE	-46.34
DICUMAROL	-3.72
DICYCLOHEXYLUREA	-14.07
DICYCLOMINE HYDROCHLORIDE	-2.08
DIDEACETYL (1,3)-7-DESACETOXY-7-OXO-DEOXYKHIVORINIC ACID	8.34
DIENESTROL	-24.92
DIETHYLCARBAMAZINE CITRATE	-4.73
DIETHYLSTILBESTROL	7.25
DIETHYLTOLUAMIDE	-1.25
DIFFRACTAIC ACID	-20.97
DIFLORASONE DIACETATE	31.48
DIFLOXACIN HYDROCHLORIDE	-6.58
DIFLUBENZURON	4.03
DIFLUNISAL	-43.34
DIFUCOL HEXAMETHYL ETHER	-3.07
DIGITONIN	102.13
DIGITOXIN	-9.00
DIGOXIGENIN	-5.99
DIGOXIN	5.01
DIHYDROCELASTROL	-123.96
DIHYDROCELASTRYL DIACETATE	-84.14
DIHYDROERGOTAMINE MESYLATE	-24.92
DIHYDROFISSINOLIDE	-45.93
DIHYDROFOLIC ACID	-16.00
DIHYDROGAMBOGIC ACID	-179.43
DIHYDROGEDUNIC ACID, METHYL ESTER	-28.41
DIHYDROGEDUNIN	-23.26
DIHYDROJASMONIC ACID	109.86
DIHYDROJASMONIC ACID, METHYL ESTER	-53.32
DIHYDROMUNDULETONE	-77.02
DIHYDROMYRISTICIN	-97.45
DIHYDROROTENONE	-102.56
DIHYDROSTREPTOMYCIN SULFATE	-8.78
DIHYDROXY (3alpha,12alpha)PREGNAN-20-ONE	-2.12

Table A.3 Continued.

DILAZEP DIHYDROCHLORIDE	-14.95
DILOXANIDE FUROATE	-34.18
DILTIAZEM HYDROCHLORIDE	-16.56
DIMENHYDRINATE	-5.60
DIMERCAPROL	-730.52
DIMETHADIONE	24.57
DIMETHYL 4,4-o-PHENYLENE-BIS (3-THIOPHANATE)	-60.23
DIMETHYL FUMARATE	-155.37
DIMETHYLCAFFEIC ACID	-4.09
DIMINAZENE ACETURATE	-6.05
DIMPYLATE	-33.09
DINITOLMIDE	-7.17
DIOSGENIN	-31.75
DIOSMETIN	11.02
DIOSMIN	-10.21
DIOXYBENZONE	-57.09
DIPERODON HYDROCHLORIDE	-9.35
DIPHENHYDRAMINE HYDROCHLORIDE	-34.31
DIPHENYLPYRALINE HYDROCHLORIDE	14.31
DIPLOSALSALATE	-12.54
DIPTERYXIN	22.49
DIPYRIDAMOLE	44.24
DIPYROCETYL	-14.95
DIPYRONE	-17.20
DIRITHROMYCIN	8.89
DISOPYRAMIDE PHOSPHATE	-3.22
DISULFIRAM	-673.59
DJENKOLIC ACID	3.40
D-LACTITOL MONOHYDRATE	-6.94
d-LIMONENE	5.90
DOBUTAMINE HYDROCHLORIDE	4.80
DOCETAXEL	-27.18
DOCOSANOL	12.46
DOCUSATE SODIUM	7.07
DOMPERIDONE	-68.51
DONEPEZIL HYDROCHLORIDE	-20.72
DOPAMINE HYDROCHLORIDE	6.01

Table A.3 Continued.

DORAMECTIN	-83.97
DOXAPRAM HYDROCHLORIDE	-11.94
DOXAZOSIN MESYLATE	-63.71
DOXEPIN HYDROCHLORIDE	12.00
DOXIFLURIDINE	-9.51
DOXOFYLLINE	10.86
DOXORUBICIN	41.45
DOXYCYCLINE HYDROCHLORIDE	38.93
DOXYLAMINE SUCCINATE	-4.51
D-PHENYLALANINE	-37.92
DROFENINE HYDROCHLORIDE	-15.03
DROPERIDOL	-32.38
DROSPIRENONE	-20.41
DUARTIN (-)	-66.38
DUARTIN, DIMETHYL ETHER	-68.02
DULOXETINE HYDROCHLORIDE	-58.70
DUTASTERIDE	-31.22
DYCLONINE HYDROCHLORIDE	24.95
DYDROGESTERONE	7.19
DYPHYLLINE	29.23
EBSELEN	-47.51
ECAMSULE TRIETHANOLAMINE	12.01
ECONAZOLE NITRATE	-79.20
EDARAVONE	-42.09
EDETATE DISODIUM	-11.09
EDITOL	-35.88
EDOXUDINE	37.66
EDROPHONIUM CHLORIDE	40.41
EFAROXAN HYDROCHLORIDE	-29.22
EFLOXATE	-208.19
ELAIDYLPHOSPHOCHOLINE	50.01
ELETRIPTAN HYDROBROMIDE	3.39
ELLAGIC ACID	-26.13
EMBELIN	-31.22
EMETINE	0.58
EMODIN	-555.53
ENALAPRIL MALEATE	-15.23

Table A.3 Continued.

ENALAPRILAT	-26.80
ENILCONAZOLE	-9.40
ENOXACIN	30.82
ENOXOLONE	-29.90
ENROFLOXACIN	8.12
ENTANDROPHRAGMIN	-35.19
EPHEDRINE (1R,2S) HYDROCHLORIDE	-6.61
EPI(13)TORULOSOL	-53.79
EPIAFZELECHIN (2R,3R)(-)	-26.27
EPIAFZELECHIN TRIMETHYL ETHER	2.88
EPIANDROSTERONE	-114.16
EPICATECHIN	-3.00
EPICATECHIN MONOGALLATE	-12.77
EPICATECHIN PENTAACETATE	-10.19
EPIESTRIOL	12.06
EPIGALLOCATECHIN	-6.04
EPIGALLOCATECHIN 3,5-DIGALLATE	14.49
EPIGALLOCATECHIN-3-MONOGALLATE	-47.93
EPINEPHRINE BITARTRATE	-59.39
EPIRUBICIN HYDROCHLORIDE	-18.88
EPITESTOSTERONE	0.13
EPOXYGEDUNIN	-66.42
EPRODISATE DISODIUM	1.21
EQUILIN	-115.39
ERDOSTEINE	-5.45
ERGOCALCIFEROL	-82.78
ERGONOVINE MALEATE	23.45
ERGOSTEROL	-51.70
ERGOSTEROL ACETATE	-46.59
ERGOTAMINE TARTRATE	-6.68
ERYTHROMYCIN	-3.90
ERYTHROMYCIN ESTOLATE	-15.95
ERYTHROMYCIN ETHYLSUCCINATE	15.39
ERYTHROSE	-0.58
ERYTHROSINE SODIUM	109.92
ESCITALOPRAM OXALATE	-64.55
ESCULETIN	20.43

Table A.3 Continued.

ESCULIN MONOHYDRATE	30.74
ESEROLINE FUMARATE	-132.98
ESOMEPRAZOLE POTASSIUM	22.98
ESTRADIOL	-33.29
ESTRADIOL ACETATE	-64.70
.ESTRADIOL BENZOATE	-164.11
ESTRADIOL CYPIONATE	-192.32
ESTRADIOL DIACETATE	-112.34
ESTRADIOL DIPROPIONATE	-133.79
ESTRADIOL METHYL ETHER	-21.80
ESTRADIOL VALERATE	-155.41
ESTRADIOL-3-SULFATE, SODIUM SALT	7.24
ESTRAGOLE	-84.80
ESTRIOL	-10.22
ESTRONE	-72.10
ESTRONE ACETATE	-390.03
ESTRONE BENZOATE	-117.50
ESTROPIPATE	3.91
ETHACRIDINE LACTATE	44.59
ETHACRYNIC ACID	-164.34
ETHAMBUTOL HYDROCHLORIDE	-14.13
ETHAMIVAN	-0.49
ETHANOLAMINE OLEATE	-22.51
ETHAVERINE HYDROCHLORIDE	-82.34
ETHINYL ESTRADIOL	-113.74
ETHIONAMIDE	-10.22
ETHIONINE	-0.93
ETHISTERONE	1.79
ETHOPROPAZINE HYDROCHLORIDE	-72.54
ETHOSUXIMIDE	9.24
ETHOTOIN	2.88
ETHOXZOLAMIDE	-3.62
ETHYL PARABEN	-49.92
ETHYNODIOL DIACETATE	-456.27
ETICLOPRIDE HYDROCHLORIDE	17.01
ETIDRONATE DISODIUM	-2.08
ETODOLAC	-16.98

Table A.3 Continued.

ETOMIDATE	-20.90
ETOPOSIDE	-8.16
EUCALYPTOL	-12.92
EUCATROPINE HYDROCHLORIDE	-30.68
EUGENOL	-165.81
EUPARIN	-147.74
EUPHOL	-177.13
EUPHOL ACETATE	-27.09
EVANS BLUE	69.06
EVOXINE	4.99
EXALAMIDE	-147.14
EXEMESTANE	-56.36
EZETIMIBE	-28.05
FAMCICLOVIR	23.17
FAMOTIDINE	-23.23
FAMPRIDINE	-12.44
FAMPROFAZONE	-140.62
FARNESOL	-85.01
FAST GREEN FCF	109.86
FASUDIL HYDROCHLORIDE	7.93
FEBUXOSTAT	-16.91
FELBINAC	-19.29
FELODIPINE	-8.61
FENBENDAZOLE	-4.73
FENBUFEN	21.84
FENBUTYRAMIDE	-85.79
FENDILINE HYDROCHLORIDE	-43.69
FENOFIBRATE	-300.63
FENOFIBRIC ACID	0.88
FENOLDIPAM MESYLATE	1.18
FENOPROFEN	-0.99
FENOTEROL HYDROBROMIDE	-22.43
FENSPIRIDE HYDROCHLORIDE	8.31
FENTHION	-85.97
FERULIC ACID	13.85
FEXOFENADINE HYDROCHLORIDE	-24.58
FILIPIN	-32.72

Table A.3 Continued.

FINASTERIDE	5.00
FIPEXIDE HYDROCHLORIDE	-6.57
FIPRONIL	-120.30
FIROCOXIB	-5.83
FISETIN	17.01
FISSINOLIDE	-74.68
FLAVANONE	-131.31
FLOPROPIONE	-23.45
FLORFENICOL	30.48
FLOXURIDINE	-29.51
FLUCONAZOLE	19.07
FLUCYTOSINE	-1.25
FLUDARABINE PHOSPHATE	24.58
FLUDROCORTISONE ACETATE	18.83
FLUFENAMIC ACID	-74.93
FlUMAZENIL	1.31
FLUMEQUINE	33.31
FLUMETHASONE	-14.34
FLUMETHAZONE PIVALATE	9.14
FLUNARIZINE HYDROCHLORIDE	-123.66
FLUNISOLIDE	-15.57
FLUNIXIN MEGLUMINE	15.37
FLUNIXIN MEGLUMINE	12.34
FLUOCINOLONE ACETONIDE	-2.65
FLUOCINONIDE	25.73
FLUORESCEIN	31.65
FLUOROMETHOLONE	0.37
FLUOROURACIL	5.39
FLUOXETINE	22.16
FLUPHENAZINE HYDROCHLORIDE	54.99
FLURANDRENOLIDE	14.98
FLURBIPROFEN	-32.48
FLUROFAMIDE	2.67
FLUROTHYL	-8.75
FLUROXENE	-10.03
FLUTAMIDE	2.02
FLUTICASONE PROPIONATE	-10.03

Table A.3 Continued.

FLUVASTATIN	-45.40
FLUVOXAMINE MALEATE	-0.90
FOLIC ACID	-17.81
FOMEPIZOLE	-9.73
FOMEPIZOLE HYDROCHLORIDE	15.83
FORMESTANE	-179.59
FORMONONETIN	16.59
FOSCARNET SODIUM	13.42
FOSFOMYCIN CALCIUM	-8.52
FOSFOSAL	5.48
FRAXIDIN METHYL ETHER	-25.75
FRIEDELIN	-7.83
FTAXILIDE	-33.71
FUCOSTANOL	31.51
FULVESTRANT	-14.34
FUMARPROTOCETRARIC ACID	-26.30
FURALTADONE	-64.84
FURAZOLIDONE	-19.90
FUROSEMIDE	24.30
FUSARIC ACID	-22.58
FUSIDIC ACID	29.23
GABAPENTIN	-6.83
GABOXADOL HYDROCHLORIDE	5.40
GADOTERIDOL	15.82
GALANGIN	-21.14
GALANGIN TRIMETHYL ETHER	-24.52
GALANTAMINE	-0.84
GALLAMINE TRIETHIODIDE	-18.43
GALLIC ACID	-5.38
GAMBOGIC ACID	-12.66
gamma-AMINOBUTYRIC ACID	-30.99
GANCICLOVIR	-16.91
GANGALEOIDIN	-97.80
GARCINOLIC ACID	-17.45
GARLICIN	-16.26
GATIFLOXACIN	16.67
GEDUNIN	-108.29

Table A.3 Continued.

GEDUNOL	-11.61
GEFITINIB	-3.90
GEMFIBROZIL	-11.26
GEMIFLOXACIN MESYLATE	30.71
GENETICIN	-2.99
GENISTEIN	-121.96
GENTAMICIN SULFATE	-13.47
GENTIAN VIOLET	110.02
GIBBERELLIC ACID	-9.32
GINKGOLIDE A	-9.92
GITOXIGENIN	-19.34
GITOXIGENIN DIACETATE	-35.60
GITOXIN	-5.19
GLAFENINE	-127.42
GLICLAZIDE	-22.37
GLIMEPIRIDE	-47.07
GLIPIZIDE	-7.23
GLUCITOL-4-GUCOPYANOSIDE	10.92
GLUCONOLACTONE	-12.77
GLUCOSAMINE HYDROCHLORIDE	-3.36
GLUCOSAMINIC ACID	-7.00
GLUTAMINE (D)	-9.28
GLUTAMINE (L)	28.35
GLUTATHIONE	-3.66
GLYBURIDE	-27.23
GLYCOCHOLIC ACID	-0.53
GLYCOPYRROLATE	10.92
GLYCYRRHIZIC ACID, AMMONIUM SALT	-0.40
GOSSYPETIN	-39.46
GOSSYPIN	1.49
GOSSYPOL	-141.55
GRAMICIDIN	-340.92
GRAMINE	8.53
GRANISETRON HYDROCHLORIDE	15.28
GRISEOFULVIN	-95.70
GUAIAZULENE	-72.68
GUAIFENESIN	-24.82

Table A.3 Continued.

GUAIOL(-)	-90.01
GUANABENZ ACETATE	12.00
GUANADREL SULFATE	8.56
GUANETHIDINE MONOSULFATE	-31.58
GUANFACINE HYDROCHLORIDE	-23.67
GUANIDINE HYDROCHLORIDE	-1.83
HAEMATOMMIC ACID	-19.34
HAEMATOMMIC ACID, ETHYL ESTER	-179.06
HAEMATOPORPHYRIN	87.76
HAEMATOXYLIN	11.48
HAEMATOXYLIN PENTAACETATE	-53.17
HALAZONE	-12.73
HALOFANTRINE HYDROCHLORIDE	-5.27
HALOPERIDOL	22.98
HALOTHANE	11.70
HARMALINE	29.19
HARMALOL HYDROCHLORIDE	41.13
HARMANE	-37.78
HARMINE	-29.14
HARMOL HYDROCHLORIDE	-7.60
HARPAGOSIDE	2.40
HECOGENIN	-14.21
HECOGENIN ACETATE	-49.92
HEDERACOSIDE C	-1.06
HEDERAGENIN	31.02
HELENINE	-230.86
HELICIN	-16.85
HEMATEIN	-18.64
HEMICHOLINIUM BROMIDE	-12.23
HEPTAMINOL HYDROCHLORIDE	-30.69
HESPERETIN	-9.62
HESPERIDIN	-37.84
HETACILLIN POTASSIUM	25.08
HETEROPEUCENIN, METHYL ETHER	-117.74
HEXACHLOROPHENE	-140.70
HEXAMETHONIUM BROMIDE	14.07
HEXAMETHYLQUERCETAGETIN	-133.71

Table A.3 Continued.

HEXESTROL	-90.33
HEXETIDINE	-81.36
HEXYLENE GLYCOL	3.01
HEXYLRESORCINOL	-20.50
HIERACIN	-10.67
HISTAMINE DIHYDROCHLORIDE	-20.24
HOMATROPINE HYDROBROMIDE	19.04
HOMATROPINE METHYLBROMIDE	14.96
HOMIDIUM BROMIDE	100.44
HOMOPTEROCARPIN	-254.80
HOMOSALATE	-244.45
HUMULENE (alpha)	-98.88
HUPERZINE A	-2.19
HYCANTHONE	34.41
HYDRALAZINE HYDROCHLORIDE	28.39
HYDRASTINE (1R, 9S)	-15.80
HYDRASTININE HYDROCHLORIDE	18.62
HYDROCHLOROTHIAZIDE	27.36
HYDROCORTISONE	-18.26
HYDROCORTISONE ACETATE	15.44
HYDROCORTISONE BUTYRATE	8.59
HYDROCORTISONE HEMISUCCINATE	-9.23
HYDROCORTISONE PHOSPHATE TRIETHYLAMINE	-4.08
HYDROCORTISONE VALERATE	-8.45
HYDROFLUMETHIAZIDE	-26.17
HYDROLYSIS PRODUCT OF BUSSEIN	7.59
HYDROQUINIDINE	12.40
HYDROQUININE HYDROBROMIDE HYDRATE	3.20
HYDROQUINONE	-23.42
HYDROXYAMPHETAMINE HYDROBROMIDE	-2.47
HYDROXYCHLOROQUINE SULFATE	8.75
HYDROXYPROGESTERONE	-9.68
HYDROXYPROGESTERONE CAPROATE	-203.26
HYDROXYTOLUIC ACID	9.78
HYDROXYUREA	3.18
HYDROXYZINE PAMOATE	18.15
HYMECHROME	37.37

Table A.3 Continued.

HYMECROMONE METHYL ETHER	-131.59
HYOSCYAMINE	7.01
HYPOXANTHINE	-1.78
IBANDRONATE SODIUM	0.33
IBUPROFEN	-6.26
ICARIIN	3.86
IDAZOXAN HYDROCHLORIDE	-45.18
IDEBENONE	-168.34
IDOXURDINE	4.84
IDRAMANTONE	-2.95
IFOSFAMIDE	-12.21
IMEXON	-30.03
IMIDAZOL-4-YLACETIC ACID SODIUM SALT	-14.96
IMIPENEM	8.23
IMIPRAMINE HYDROCHLORIDE	-68.34
IMIQUIMOD	-26.25
INDAPAMIDE	2.79
INDOLE-3-CARBINOL	-41.80
INDOMETHACIN	13.76
INDOPROFEN	6.76
INOSINE	-28.64
INOSITOL	-15.30
IODIPAMIDE	-32.38
IODIXANOL	-2.51
IODOQUINOL	15.50
IOHEXOL	-10.58
IOPANIC ACID	-18.54
IOTHALAMIC ACID	-18.61
IOVERSOL	17.51
IOXILAN	1.73
IPRATROPIUM BROMIDE	-15.81
IPRIFLAVONE	-13.23
IPRONIAZID SULFATE	-5.60
IRBESARTAN	4.74
IRETOL	-6.70
IRIDIN	-28.76
IRIGENIN	-50.85

Table A.3 Continued.

IRIGENIN TRIMETHYL ETHER	-18.01
IRIGENIN, 7-BENZYL ETHER	-83.12
IRIGENIN, DIBENZYL ETHER	-29.04
IRIGENOL	-54.16
IRIGINOL HEXAACEATATE	-6.05
IRINOTECAN HYDROCHLORIDE	23.40
IRSOGLADINE MALEATE	-10.52
ISAXONINE	-46.23
ISOBERGAPTENE	-0.12
ISOBUTAMBEN	-64.42
ISOBUTYLMETHYLXANTHINE	-46.70
ISOETHARINE MESYLATE	-108.48
ISOFLUPREDNONE ACETATE	-81.16
ISOGINKGETIN	1.33
ISOKOBUSONE	4.16
ISOLIQUIRITIGENIN	-35.09
ISONIAZID	4.13
ISOPEONOL	-2.73
ISOPIMPINELLIN	-73.27
ISOPROPAMIDE IODIDE	10.64
ISOPROTERENOL HYDROCHLORIDE	-20.94
ISOROTENONE	-71.23
ISOSAFROLE	-28.02
ISOSORBIDE DINITRATE	30.36
ISOSORBIDE MONONITRATE	-14.39
ISOTECTORIGENIN, 7-METHYL ETHER	-15.39
ISOTRETINON	-80.73
ISOVALERAMIDE	5.71
ISOXICAM	3.20
ISOXSUPRINE HYDROCHLORIDE	-34.31
ISRADIPINE	-51.71
ITOPRIDE HYDROCHLORIDE	0.24
ITRACONAZOLE	-114.11
IVERMECTIN	-151.99
JUAREZIC ACID	-20.65
JUGLONE	-99.47
KAEMPFEROL	10.36

Table A.3 Continued.

KAINIC ACID	3.28
KANAMYCIN A SULFATE	36.98
KARANJIN	7.64
KASUGAMYCIN HYDROCHLORIDE	14.29
KAWAIN	27.60
KETANSERIN TARTRATE	-6.71
KETOCONAZOLE	-241.68
KETOPROFEN	-13.29
KETOROLAC TROMETHAMINE	0.65
KETOTIFEN FUMARATE	-23.97
KHAYANTHONE	-38.72
KHELLIN	-24.00
KHIVORIN	-24.41
KINETIN	25.83
KINETIN RIBOSIDE	-54.96
KOBUSONE	18.62
KOPARIN	-49.17
KYNURENINE	-38.26
L(+/-)-ALLIIN	-10.90
LABETALOL HYDROCHLORIDE	-4.26
LACCAIC ACID A	7.53
LACTOBIONIC ACID	-7.22
LACTULOSE	24.34
LAGOCHILIN	-12.35
LAMIVUDINE	-13.49
LAMOTRIGINE	-10.35
LANATOSIDE C	-31.92
LANOSTEROL	-44.71
LANOSTEROL ACETATE	-85.79
LANSOPRAZOLE	-68.21
LAPPACONITINE	-14.14
LARIXINIC ACID	-30.44
LARIXOL	-82.35
LARIXOL ACETATE	-125.41
LATHOSTEROL	-6.26
L-BUTHIONINE SULFOXIMINE	-16.80
L-DEOXYALLIIN	-15.68

Table A.3 Continued.

LECANORIC ACID	21.37
LEFLUNOMIDE	6.27
LEOIDIN	-164.28
LETROZOLE	-0.62
LEUCOVORIN CALCIUM	-10.22
LEVALBUTEROL HYDROCHLORIDE	-14.95
LEVAMISOLE HYDROCHLORIDE	-18.37
LEVCYCLOSERINE	-6.98
LEVOBUNOLOL HYDROCHLORIDE	-21.02
LEVOCARNITINE	7.57
LEVOCARNITINE PROPIONATE HYDROCHLORIDE	5.20
LEVOCETIRIZINE DIHYDROCHLORIDE	-14.69
LEVODOPA	-8.69
LEVOFLOXACIN	23.45
LEVOMENTHOL	-28.64
LEVONORDEFRIN	-31.08
LEVONORGESTREL	-64.46
LEVOSIMENDAN	-31.57
LEVOTHYROXINE	3.01
LEVULINIC ACID, 3-BENZYLIDENYL-	-7.15
LIDOCAINE HYDROCHLORIDE	-23.59
LIDOFLAZINE	13.06
LIGUSTILIDE	-110.50
LINAMARIN	-6.43
LINCOMYCIN HYDROCHLORIDE	-4.29
LINDANE	-3.14
LIOTHYRONINE	2.87
LIOTHYRONINE (L- isomer) SODIUM	0.18
LIPOAMIDE	-24.27
LISINOPRIL	21.21
LITHIUM CITRATE	24.58
LITHOCHOLIC ACID	-89.59
L-LEUCYL-L-ALANINE	16.25
LOBARIC ACID	-1.18
LOBELINE HYDROCHLORIDE	-7.17
LOBENDAZOLE	-45.05
LOFEXIDINE HYDROCHLORIDE	6.57

Table A.3 Continued.

LOMEFLOXACIN HYDROCHLORIDE	-37.74
LOMERIZINE HYDROCHLORIDE	-255.69
LOMUSTINE	-41.80
LONIDAMINE	-24.45
LOPERAMIDE HYDROCHLORIDE	30.36
LORATADINE	38.17
LORGLUMIDE SODIUM	-8.23
LORNOXICAM	-8.90
LOSARTAN	-13.12
LOVASTATIN	-44.37
LOXAPINE SUCCINATE	-2.15
L-PHENYLALANINOL	-18.64
LUNARINE	-22.90
LUPANINE PERCHLORATE	11.09
LUPANYL ACID HYDROCHLORIDE	-18.30
LUPEOL	6.30
LUPININE	3.10
MADECASSIC ACID	-6.57
MAFENIDE HYDROCHLORIDE	-44.13
MALATHION	-476.24
MANGAFODIPIR TRISODIUM	-1.04
MANGANESE TETRAKIS(4-CARBOXYPHENYL)PORPHYRIN CHLORDE	-5.97
MANGIFERIN	16.86
MANGOSTIN TRIMETHYL ETHER	-45.49
MANNITOL	-3.72
MAPROTILINE HYDROCHLORIDE	5.83
MEBENDAZOLE	0.62
MEBEVERINE HYDROCHLORIDE	40.75
MEBHYDROLIN NAPHTHALENESULFONATE	-39.97
MECAMYLAMINE HYDROCHLORIDE	21.30
MECHLORETHAMINE	-23.75
MECLIZINE HYDROCHLORIDE	-472.39
MECLOCYCLINE SULFOSALICYLATE	19.17
MECLOFENAMATE SODIUM	-53.54
MECLOFENOXATE HYDROCHLORIDE	-0.03
MECYSTEINE HYDROCHLORIDE	-15.61
MEDROXYPROGESTERONE ACETATE	-3.00

Table A.3 Continued.

MEDRYSONE	-54.33
MEFENAMIC ACID	-104.16
MEFEXAMIDE	4.94
MEFLOQUINE	53.47
MEGESTROL ACETATE	-54.20
MEGLUMINE	-17.08
MEGLUTOL	-20.59
MELATONIN	-11.51
MELENGESTROL ACETATE	-110.87
MELEZITOSE	9.79
MELIBIOSE	-19.24
MELOXICAM SODIUM	-11.27
MELPERONE HYDROCHLORIDE	-23.27
MELPHALAN	-19.17
MEMANTINE HYDROCHLORIDE	-14.15
MENADIONE	93.00
MENAQUINONE-4	-99.29
MENTHONE	-18.49
MENTHYL BENZOATE	-1200.51
MEPARTRICIN	-66.68
MEPENZOLATE BROMIDE	-41.62
MEPHENESIN	8.44
MEPHENTERMINE SULFATE	-8.14
MEPIROXOL	-2.46
MEPIVACAINE HYDROCHLORIDE	-22.30
MEPRYLCAINE HYDROCHLORIDE	16.60
MEQUINOL	-65.26
MERBROMIN	105.70
MERCAPTOPURINE	-13.91
MEROGEDUNIN	-12.66
MEROPENEM	31.22
MESALAMINE	27.95
MESNA	22.55
MESORIDAZINE BESYLATE	2.80
MESTRANOL	-30.72
METACETAMOL	4.89
meta-CRESYL ACETATE	5.01

Table A.3 Continued.

METAMECONINE	-23.12
METAMPICILLIN SODIUM	-11.38
METAPROTERENOL	-1.43
METARAMINOL BITARTRATE	-4.90
METAXALONE	-41.43
METERGOLINE	-256.18
METFORMIN HYDROCHLORIDE	-9.80
METHACHOLINE CHLORIDE	-24.46
METHACYCLINE HYDROCHLORIDE	10.21
METHAPYRILENE HYDROCHLORIDE	2.22
METHAZOLAMIDE	-11.04
METHENAMINE	1.06
METHICILLIN SODIUM	23.03
METHIMAZOLE	13.78
METHIONINE SULFOXIMINE (L)	-6.34
METHOCARBAMOL	-8.77
METHOPRENE (S)	-758.04
METHOTREXATE(+/-)	23.75
METHOXAMINE HYDROCHLORIDE	15.54
METHOXSALEN	-74.98
METHOXYAMINE HYDROCHLORIDE	-0.38
METHOXYVONE	-140.43
METHSCOPOLAMINE BROMIDE	33.82
METHSUXIMIDE	-26.05
METHYCLOTHIAZIDE	-6.98
METHYL 7-DESHYDROXYPYROGALLIN-4-CARBOXYLATE	-75.60
METHYL DEOXYCHOLATE	-157.39
METHYL ORSELLINATE	-22.37
METHYL ROBUSTONE	-87.88
METHYLATROPINE NITRATE	-1.45
METHYLBENZETHONIUM CHLORIDE	-55.73
METHYLDOPA	-24.06
METHYLDOPATE HYDROCHLORIDE	5.92
METHYLENE BLUE	109.92
METHYLERGONOVINE MALEATE	34.97
METHYLPHENIDATE HYDROCHLORIDE	1.85
METHYLPREDNISOLONE	0.70

Table A.3 Continued.

METHYLPREDNISOLONE SODIUM SUCCINATE	9.31
METHYLTHIOURACIL	-15.30
METHYLXANTHOXYLIN	-72.09
METHYSERGIDE MALEATE	-11.51
METICRANE	0.17
METITEPINE MALEATE	46.44
METOCLOPRAMIDE HYDROCHLORIDE	-1.81
METOLAZONE	25.13
METOPROLOL TARTRATE	-14.90
METRONIDAZOLE	-31.68
METYRAPONE	2.50
MEVALONIC ACID LACTONE	15.79
MEVASTATIN	0.17
MEXAMINE	-27.47
MEXICANOLIDE	-20.56
MEXILETINE HYDROCHLORIDE	-11.20
MIANSERIN HYDROCHLORIDE	14.01
MICONAZOLE NITRATE	-197.94
MIDODRINE HYDROCHLORIDE	23.55
MIFEPRISTONE	-106.01
MIGLITOL	8.77
MILNACIPRAN HYDROCHLORIDE	7.80
MILRINONE	1.70
MILTEFOSINE	-7.55
MIMOSINE	18.62
MINAPRINE HYDROCHLORIDE	11.90
MINOCYCLINE HYDROCHLORIDE	-30.78
MINOXIDIL	-11.56
MITOMYCIN	-101.21
MITOTANE	-747.79
MITOXANTRONE HYDROCHLORIDE	97.53
MOCLOBEMIDE	-5.01
MODAFINIL	-2.86
MODALINE SULFATE	-57.43
MOGUISTEINE	-19.08
MOLINDONE HYDROCHLORIDE	-0.92
MOLSIDOMINE	-2.93

Table A.3 Continued.

MONENSIN SODIUM (monensin A is shown)	-84.16
MONOBENZONE	-86.14
MONOCROTALINE	-1.45
MONTELUKAST SODIUM	8.11
MORANTEL CITRATE	34.75
MORIN	0.91
MOROXYDINE HYDROCHLORIDE	25.64
MOXALACTAM DISODIUM	3.97
MOXIDECTIN	-131.04
MOXIFLOXACIN HYDROCHLORIDE	48.63
MOXISYLYTE HYDROCHORIDE	-7.47
MUCIC ACID	-12.97
MUNDOSERONE	-131.36
MUNDULONE	-117.53
MUNDULONE ACETATE	-291.40
MUPIROCIN	11.17
MUUROLLADIE-3-ONE	16.43
MYCOPHENOLATE MOFETIL	-14.40
MYCOPHENOLIC ACID	0.02
MYOSMINE	-35.34
N- (9-FLUORENYLMETHOXYCARBONYL)-L-LEUCINE	-75.94
N,N-HEXAMETHYLENEAMILORIDE	-13.84
NABUMETONE	-4.83
N-ACETYLMURAMIC ACID	-6.11
N-ACETYLNEURAMIC ACID	-16.08
N-ACETYLPROLINE	-4.24
NADIDE	-40.00
NADIFLOXACIN	30.42
NADOLOL	6.23
NAFCILLIN SODIUM	12.97
NAFRONYL OXALATE	16.04
NAFTIFINE HYDROCHLORIDE	-63.86
NAFTOPIDIL	-39.21
NALBUPHINE HYDROCHLORIDE	8.53
NALIDIXIC ACID	7.37
NALOXONE HYDROCHLORIDE	-4.61
NALTREXONE HYDROCHLORIDE	-5.04

Table A.3 Continued.

NAPHAZOLINE HYDROCHLORIDE	1.77
NAPROXEN(+)	-1.43
NAPROXOL	-11.58
NARINGENIN	-5.91
NARINGIN	10.47
NATAMYCIN	23.92
NATEGLINIDE	0.82
NEFAZODONE HYDROCHLORIDE	-55.26
NEFOPAM	-7.51
NELARABIN	0.70
NEOMYCIN SULFATE	-6.70
NEOSTIGMINE BROMIDE	3.84
NEROL	-49.31
NEROLIDOL	-12.09
NETILMICIN SULFATE	18.89
NEVIRAPINE	-8.01
NIACIN	-27.07
NIACINAMIDE	12.40
NIALAMIDE	-16.94
NICARDIPINE HYDROCHLORIDE	-510.40
NICERGOLINE	-57.00
NICLOSAMIDE	-24.31
NICORANDIL	20.33
NICOTINE BITARTRATE	-23.06
NICOTINYL ALCOHOL TARTRATE	-29.41
NIFEDIPINE	-125.76
NIFENAZONE	-8.94
NIFLUMIC ACID	-53.03
NIFUROXAZIDE	-141.38
NIFURSOL	-61.92
NIKETHAMIDE	11.15
NILUTAMIDE	-24.65
NIMESULIDE	-104.07
NIMODIPINE	-36.16
NIMUSTINE	-37.07
NIPECOTIC ACID	-4.02
NISOLDIPINE	-238.72

Table A.3 Continued.

NITARSONE	19.99
NITAZOXANIDE	-61.10
NITHIAMIDE	-3.90
NITRENDIPINE	-29.12
NITROFURANTOIN	-12.08
NITROFURAZONE	-18.43
NITROMIDE	-23.78
NITROXOLINE	-85.47
NIZATIDINE	-4.29
N-METHYL (-)EPHEDRINE [1R,2S]	-3.64
N-METHYLANTHRANILIC ACID	24.37
N-METHYLBENZYLAMINE HYDROCHLORIDE	3.33
N-METHYL-D-ASPARTIC ACID (NMDA)	-16.48
N-METHYLISOLEUCINE	-6.56
NOBILETIN	-101.77
NOCODAZOLE	-32.72
NOMIFENSINE MALEATE	2.88
NONIC ACID	-1.85
NONOXYNOL-9	-58.57
NORCANTHARIDIN	-56.65
NOREPINEPHRINE	-12.25
NORETHINDRONE	-56.45
NORETHINDRONE ACETATE	-70.94
NORETHYNODREL	-49.40
NORFLOXACIN	20.84
NORGESTIMATE	-472.86
NORGESTREL	-21.03
NORHARMAN	-34.27
NORSTICTIC ACID	18.31
NORSTICTIC ACID PENTAACETATE	-184.67
NORTRIPTYLINE	7.73
NOSCAPINE HYDROCHLORIDE	-19.20
NOVOBIOCIN SODIUM	-2.40
N-PHENYLANTHRANILIC ACID	-33.90
NYLIDRIN HYDROCHLORIDE	-16.91
NYSTATIN	-8.55
O-BENZYL-l-SERINE	-4.09

Table A.3 Continued.

OBLIQUIN	-45.82
OBTUSAQUINONE	-1452.63
OCTISALATE	-156.78
OCTOCRYLENE	-218.18
OCTODRINE	17.98
OCTOPAMINE HYDROCHLORIDE	21.70
OFLOXACIN	37.31
OLEANDOMYCIN PHOSPHATE	8.47
OLEANOIC ACID	-30.95
OLEANOLIC ACID ACETATE	-66.81
OLIGOMYCIN (A shown)	-105.05
OLMESARTAN	16.99
OLMESARTAN MEDOXOMIL	-12.92
OLTIPRAZ	-207.03
OMEPRAZOLE	-2.93
ONDANSETRON	-0.55
ORBIFLOXACIN	0.85
ORLISTAT	-68.21
ORNIDAZOLE	-27.00
ORNITHINE	10.90
OROTIC ACID	-15.03
ORPHENADRINE CITRATE	3.05
ORSELLINIC ACID	-0.78
ORSELLINIC ACID, ETHYL ESTER	-64.14
OSAJIN	-526.77
OSELTAMIVIR PHOSPHATE	24.61
OUABAIN	-11.67
o-VERATRALDEHYDE	-76.02
OXACILLIN SODIUM	-7.35
OXALIPLATIN	5.85
OXANTEL PAMOATE	20.56
OXAPROZIN	-2.36
OXCARBAZEPINE	-18.29
OXEDRINE	-18.82
OXELAIDIN CITRATE	-10.52
OXETHAZAINE	-43.00
OXFENDAZOLE	-11.66

Table A.3 Continued.

OXIBENDAZOLE	6.35
OXICONAZOLE NITRATE	-187.67
OXIDOPAMINE HYDROCHLORIDE	-9.98
OXIGLUTATIONE DISODIUM SALT	13.68
OXOLAMINE CITRATE	-81.85
OXOLINIC ACID	22.37
OXONITINE	-5.41
OXTRIPHYLLINE	-16.15
OXYBENZONE	-58.72
OXYBUTYNIN CHLORIDE	-12.42
OXYMETAZOLINE HYDROCHLORIDE	8.29
OXYPHENBUTAZONE	-171.53
OXYPHENCYCLIMINE HYDROCHLORIDE	-11.24
OXYPHENONIUM BROMIDE	-7.47
OXYQUINOLINE SULFATE	-28.80
OXYTETRACYCLINE	3.89
OXYTHIAMINE CHLORIDE HYDROCHLORIDE	-11.15
OZAGREL HYDROCHLORIDE	7.23
PACHYRRHIZIN	14.76
PACLITAXEL	-10.58
PAEONOL	-7.59
PALIPERIDONE	-5.50
PALMATINE	35.20
PALMATINE CHLORIDE	31.18
PANCURONIUM BROMIDE	14.30
PANGAMIC ACID SODIUM	-5.70
PANTETHINE	-14.86
PANTHENOL (dl)	-7.04
PANTOPRAZOLE	-11.99
PANTOTHENIC ACID(d) Na salt	-19.44
PAPAVERINE HYDROCHLORIDE	-73.90
PARACHLOROPHENOL	-11.74
PARAMETHADIONE	23.82
PARAROSANILINE PAMOATE	-79.76
PARAXANTHINE	-38.05
PARGYLINE HYDROCHLORIDE	19.63
PAROMOMYCIN SULFATE	6.78

Table A.3 Continued.

PAROXETINE HYDROCHLORIDE	44.58
PASINIAZID	1.45
PATULIN	-342.17
PAZUFLOXACIN MESYLATE	-27.09
p-CHLOROPHENYLALANINE	17.71
PECTOLINARIN	-40.94
PEFLOXACINE MESYLATE	15.01
PELLETIERINE HYDROCHLORIDE	-27.74
PEMPIDINE TARTRATE	-33.40
PENBUTOLOL SULFATE	-27.87
PENCICLOVIR	2.47
PENFLURIDOL	-134.52
PENICILLAMINE	3.45
PENICILLIN G POTASSIUM	-29.00
PENICILLIN V POTASSIUM	8.29
PENTACHLOROPHENOL	-872.61
PENTAGASTRIN	-4.22
PENTAMIDINE ISETHIONATE	-20.93
PENTETIC ACID	25.54
PENTOLINIUM TARTRATE	-7.49
PENTOXIFYLLINE	4.80
PENTYLENETETRAZOL	3.75
PEONIFLORIN	-16.85
PERGOLIDE MESYLATE	-21.16
PERHEXILINE MALEATE	28.96
PERICIAZINE	-2.39
PERILLIC ACID (-)	-21.83
PERILLYL ALCOHOL	-94.09
PERINDOPRIL ERBUMINE	13.69
PERMETHRIN	-103.25
PERPHENAZINE	66.40
PERSEITOL	8.23
PERSEITOL HEPTAACETATE	8.17
PERUVOSIDE	-10.68
PEUCENIN	-12.05
p-FLUOROPHENYLALANINE	-10.99
PHENACEMIDE	15.88

Table A.3 Continued.

PHENACETIN	-33.29
PHENACYLAMINE HYDROCHLORIDE	-24.50
PHENAZOPYRIDINE HYDROCHLORIDE	-10.03
PHENELZINE SULFATE	3.71
PHENETHYL CAFFEATE (CAPE)	-137.03
PHENFORMIN HYDROCHLORIDE	8.17
PHENINDIONE	2.40
PHENIRAMINE MALEATE	-11.09
PHENOLPHTHALEIN	-34.52
PHENOTHIAZINE	-186.10
PHENOTHRIN	-151.67
PHENOXYBENZAMINE HYDROCHLORIDE	-324.11
PHENSUCCIMIDE	-5.96
PHENTERMINE	-28.55
PHENTOLAMINE HYDROCHLORIDE	-0.05
PHENYL AMINOSALICYLATE	18.80
PHENYLBUTAZONE	-1.95
PHENYLBUTYRATE SODIUM	-19.51
PHENYLEPHRINE HYDROCHLORIDE	-24.92
PHENYLETHYL ALCOHOL	15.98
PHENYLMERCURIC ACETATE	101.37
PHENYLPROPANOLAMINE HYDROCHLORIDE	3.32
PHENYTOIN SODIUM	-25.36
PHLORACETOPHENONE	-6.78
PHLORETIN	-121.32
PHLORIDZIN	19.65
PHTHALYLSULFACETAMIDE	-5.38
PHTHALYLSULFATHIAZOLE	-8.37
PHYSCION	-484.54
PHYSOSTIGMINE SALICYLATE	-3.29
PHYTOL	-184.67
PHYTONADIONE	-29.46
PICEID	-24.10
PICROPODOPHYLLIN	-48.28
PICROPODOPHYLLIN ACETATE	-10.90
PICROTIN	-5.12
PICROTOXININ	3.04

Table A.3 Continued.

PIDOTIMOD	-18.73
PILOCARPINE NITRATE	-10.38
PIMAGEDINE HYDROCHLORIDE	-7.49
PIMETHIXENE MALEATE	9.60
PIMOZIDE	-275.76
PIMPINELLIN	-33.80
PINACIDIL	5.20
PINDOLOL	6.33
PIOGLITAZONE HYDROCHLORIDE	-25.59
PIPAMPERONE	27.69
PIPEMIDIC ACID	-3.73
PIPENZOLATE BROMIDE	-1.62
PIPERACETAZINE	32.62
PIPERACILLIN SODIUM	4.76
PIPERAZINE	-14.30
PIPERIC ACID	5.62
PIPERIDOLATE HYDROCHLORIDE	16.41
PIPERINE	6.20
PIPERONYL BUTOXIDE	-7.37
PIPERONYLIC ACID	4.81
PIPLARTINE	-131.31
PIPOBROMAN	47.44
PIRACETAM	2.54
PIRENPERONE	-37.75
PIRENZEPINE HYDROCHLORIDE	4.84
PIRIBEDIL HYDROCHLORIDE	-4.73
PIROCTONE OLAMINE	-54.55
PIROMIDIC ACID	1.91
PIROXICAM	3.81
PISCIDIC ACID	-4.43
PIZOTYLINE MALATE	3.52
PLUMBAGIN	-23.88
PODOFILOX	-7.28
PODOPHYLLIN ACETATE	-83.68
PODOTOTARIN	-13.20
POLYMYXIN B SULFATE	9.80
POMIFERIN	-366.96

Table A.3 Continued.

POTASSIUM p-AMINOBENZOATE	-21.37
PRALIDOXIME CHLORIDE	29.77
PRALIDOXIME MESYLATE	11.52
PRAMIPEXOLE DIHYDROCHLORIDE	14.42
PRAMOXINE HYDROCHLORIDE	-1.39
PRANOPROFEN	0.85
PRASTERONE	-17.36
PRASTERONE ACETATE	-107.41
PRASUGREL	-19.55
PRAVASTATIN SODIUM	8.69
PRAZIQUANTEL	-2.57
PRAZOSIN HYDROCHLORIDE	-2.15
PREDNICARBATE	-21.54
PREDNISOLONE	6.08
PREDNISOLONE ACETATE	-23.41
PREDNISOLONE HEMISUCCINATE	-6.56
PREDNISOLONE SODIUM PHOSPHATE	16.46
PREDNISONE	-8.70
PREGABALIN	1.18
PREGNENOLONE SUCCINATE	7.57
PRENYLETIN	14.18
PRIDINOL METHANESULFONATE	13.74
PRILOCAINE HYDROCHLORIDE	-5.04
PRIMAQUINE PHOSPHATE	-80.31
PRIMIDONE	-7.44
PRIMULETIN	-140.80
PRISTIMERIN	-166.52
PROADIFEN HYDROCHLORIDE	-9.73
PROBENECID	-36.27
PROBUCOL	22.18
PROCAINAMIDE HYDROCHLORIDE	2.54
PROCAINE HYDROCHLORIDE	-3.50
PROCARBAZINE HYDROCHLORIDE	-1.60
PROCHLORPERAZINE EDISYLATE	31.52
PROCYCLIDINE HYDROCHLORIDE	-15.13
PROFLAVINE HEMISULFATE	58.47
PROGESTERONE	-113.08

Table A.3 Continued.

PROGLUMIDE	-23.71
PROMAZINE HYDROCHLORIDE	28.74
PROMETHAZINE HYDROCHLORIDE	-27.00
PRONETALOL HYDROCHLORIDE	-18.38
PROPAFENONE HYDROCHLORIDE	13.15
PROPANTHELINE BROMIDE	-50.68
PROPARACAINE HYDROCHLORIDE	-0.62
PROPENTOFYLLINE	3.28
PROPIOLACTONE	-7.35
PROPOFOL	-70.70
PROPOXUR	-23.18
PROPOXYCAINE HYDROCHLORIDE	-10.51
PROPRANOLOL HYDROCHLORIDE (+/-)	17.49
PROPYLTHIOURACIL	-9.23
PROSCILLARIDIN	-9.05
PROTIONAMIDE	3.92
PROTIRELIN	-5.37
PROTOPORPHYRIN IX	55.79
PROTOVERATRINE A	1.31
PROTRYPTYLINE HYDROCHLORIDE	26.94
PROXYPHYLLINE	5.52
PRULIFLOXACIN	-10.14
PSEUDO-ANISATIN	5.84
PSEUDOEPHEDRINE HYDROCHLORIDE	-9.68
PTAEROXYLIN	-225.22
PUERARIN	13.05
PUROMYCIN HYDROCHLORIDE	-7.27
PURPURIN	-122.18
PURPUROGALLIN	-49.43
PURPUROGALLIN-4-CARBOXYLIC ACID	-28.05
PUTRESCINE DIHYDROCHLORIDE	-15.12
PYRANTEL PAMOATE	24.39
PYRAZINAMIDE	-3.07
PYRETHRINS	-447.81
PYRIDOSTIGMINE BROMIDE	7.48
PYRIDOXINE	-1.32
PYRILAMINE MALEATE	-22.79

Table A.3 Continued.

PYRIMETHAMINE	31.31
PYRITHIONE ZINC	69.94
PYRITHYLDIONE	4.39
PYRITINOL	-14.86
PYROCATECHUIC ACID	2.63
PYROGALLIN	-345.99
PYRONARIDINE TETRAPHOSPHATE	24.19
PYRROMYCIN	-207.03
PYRVINIUM PAMOATE	-41.27
QUASSIN	-20.56
QUEBRACHITOL	-31.55
QUERCETIN	-92.20
QUERCETIN 5,7,3',4'-TETRAMETHYL ETHER	-57.93
QUERCITRIN	-4.04
QUETIAPINE	12.75
QUINACRINE HYDROCHLORIDE	50.63
QUINALIZARIN	9.84
QUINAPRIL HYDROCHLORIDE	-18.88
QUINAPRILAT	21.98
QUINESTROL	-119.97
QUINETHAZONE	0.25
QUINIC ACID	-3.88
QUINIDINE GLUCONATE	-0.46
QUININE ETHYL CARBONATE	-32.47
QUININE SULFATE	-5.53
QUINOLINIC ACID	-8.72
QUINPIROLE HYDROCHLORIDE	-16.82
QUIPAZINE MALEATE	5.50
RABEPRAZOLE SODIUM	-2.93
RACEPHEDRINE HYDROCHLORIDE	-2.16
RACTOPAMINE HYDROCHLORIDE	-18.52
RALOXIFENE HYDROCHLORIDE	-8.44
RAMIFENAZONE	10.31
RAMIPRIL	1.53
RAMOPLANIN [A2 shown; 2mM]	-110.63
RANITIDINE	-36.29
RANOLAZINE	0.19

Table A.3 Continued.

RASAGILINE	-20.07
RAUWOLSCINE HYDROCHLORIDE	7.83
REBAMIPIDE	18.98
REPAGLINIDE	4.34
RESERPINE	11.94
RESORCINOL	-3.86
RESORCINOL MONOACETATE	1.77
RESVERATROL	-4.68
RESVERATROL 4'-METHYL ETHER	-33.46
RETINOL	-70.56
RETINYL ACETATE	-182.40
RETINYL PALMITATE	-23.76
RETUSIN	3.61
RETUSIN 7-METHYL ETHER	-7.47
RHETSININE	-16.10
RHIZOCARPIC ACID	-30.07
RHODINYL ACETATE	-194.45
RHODOCLADONIC ACID	4.74
RHOIFOLIN	-4.61
RIBAVIRIN	-19.21
RIBOFLAVIN	69.85
RIBOFLAVIN 5-PHOSPHATE SODIUM	-94.57
RIBOSTAMYCIN SULFATE	-13.80
RIFAMPIN	-7.66
RIFAXIMIN	-8.06
RILUZOLE	-39.32
RIMANTADINE HYDROCHLORIDE	15.34
RISEDRONATE SODIUM	-0.83
RISPERIDONE	-36.06
RITANSERIN	-15.04
RITODRINE HYDROCHLORIDE	-16.57
RITONAVIR	1.31
RIVASTIGMINE TARTRATE	95.31
RIZATRIPTAN BENZOATE	2.55
ROBUSTIC ACID	75.72
ROCCELLIC ACID	-17.28
ROFECOXIB	-10.27

Table A.3 Continued.

ROLIPRAM	-120.30
ROLITETRACYCLINE	-36.48
RONIDAZOLE	-32.72
ROPINIROLE HYDROCHLORIDE	3.45
ROSIGLITAZONE MALEATE	10.08
ROSMARINIC ACID	7.41
ROSOLIC ACID	-57.04
ROSUVASTATIN CALCIUM	-27.38
ROTENONE	-45.82
ROTENONIC ACID, METHYL ETHER	-138.44
ROXARSONE	-18.60
ROXATIDINE ACETATE HYDROCHLORIDE	-21.81
ROXITHROMYCIN	1.04
RUFLOXACIN HYDROCHLORIDE	22.86
RUTILANTINONE	-696.28
RUTIN	-0.69
SACCHARIN	11.51
SAFROLE	-102.39
SALICIN	-42.43
SALICYL ALCOHOL	-14.71
SALICYLAMIDE	-3.65
SALICYLANILIDE	-204.66
SALIDROSIDE	19.89
SALINOMYCIN, SODIUM	-152.61
SALMETEROL XINAFOATE	-71.41
SALSALATE	16.55
SALSOLIDINE	8.47
SALSOLINE	1.82
SALSOLINOL HYDROBROMIDE	-4.64
SALVINORIN A	-105.89
SANGUINARIUM SULFATE	-38.43
SANTONIN	-30.35
SAPPANONE A DIMETHYL ETHER	-113.75
SARAFLOXACIN HYDROCHLORIDE	-6.79
SAXAGLIPTIN	11.70
SCLAREOL	-29.41
SCLAREOLIDE	-140.92

Table A.3 Continued.

SCOPOLAMINE HYDROBROMIDE	-1.32
SCOPOLETIN	24.78
SECNIDAZOLE	-11.38
SECURININE	-79.90
SELAMECTIN	-158.83
SELEGILINE HYDROCHLORIDE	-34.14
SEMUSTINE	-108.88
SENNOSIDE A	1.40
SENNOSIDE B	-20.10
SERATRODAST	-99.16
SEROTONIN HYDROCHLORIDE	2.01
SERTRALINE HYDROCHLORIDE	6.11
SHIKIMIC ACID	-8.07
SIBUTRAMINE HYDROCHLORIDE	-44.14
SILDENAFIL CITRATE	-0.55
SILIBININ	-245.16
SIMVASTATIN	-128.76
SINAPIC ACID	25.53
SINAPIC ACID METHYL ETHER	8.53
SINENSETIN	0.52
SINOMENINE	-15.70
SIROLIMUS	-77.69
S-ISOCORYDINE (+)	2.65
SISOMICIN SULFATE	-32.18
SITAGLIPTIN	-51.49
SITOSTERYL ACETATE	-12.12
SKATOLE	-83.28
SMILAGENIN	-60.25
SMILAGENIN ACETATE	-16.36
SNAP (S-NITROSO-N-ACETYLPENICILLAMINE)	9.61
SODIUM FLUOROACETATE	-43.62
SODIUM MONOFLUOROPHOSPHATE	5.21
SODIUM NITROPRUSSIDE	-0.83
SODIUM OXYBATE	-0.48
SODIUM PHENYLACETATE	-68.10
SODIUM PHENYLBUTYRATE	13.28
SODIUM SALICYLATE	-10.78

Table A.3 Continued.

SODIUM TETRADECYL SULFATE	-6.12
SODIUM THIOGLYCOLATE	27.91
SOLANESOL	-115.03
SOLANESYL ACETATE	-28.68
SOLASODINE	0.52
SOLIDAGENONE	-94.47
SOLIFENACIN SUCCINATE	-37.43
SORBITOL	-18.61
SOTALOL HYDROCHLORIDE	-1.25
SPAGLUMIC ACID	20.33
SPARFLOXACIN	0.28
SPARTEINE SULFATE	-2.02
SPECTINOMYCIN HYDROCHLORIDE	9.80
SPERMIDINE TRIHYDROCHLORIDE	-24.10
SPERMINE	-16.94
SPHONDIN	-11.84
SPIPERONE	-29.41
SPIRAMYCIN	-0.56
SPIRONOLACTONE	-28.41
SR-2640	-28.46
STAVUDINE	2.94
STICTIC ACID	6.65
STIGMASTA-4,22-DIEN-3-ONE	-16.94
STIGMASTEROL	-18.30
STREPTOMYCIN SULFATE	-11.59
STREPTOZOSIN	-1.87
STROPHANTHIDIN	-20.97
STROPHANTHIDINIC ACID LACTONE ACETATE	6.98
STRYCHNINE	10.88
STRYCHNINE METHIODIDE	15.79
SUCCINYLSULFATHIAZOLE	5.13
SUCRALFATE SODIUM (10mM 10% aq DMSO)	-58.78
SUCRALOSE	10.98
SULBACTAM	10.92
SULBENTINE	-79.20
SULCONAZOLE NITRATE	-72.73
SULFABENZAMIDE	-4.00

Table A.3 Continued.

SULFACARBAMIDE	-7.37
SULFACETAMIDE	11.64
SULFACHLORPYRIDAZINE	-35.01
SULFADIAZINE	-4.51
SULFADIMETHOXINE	-19.07
SULFADOXINE	14.01
SULFAGUANIDINE	-8.15
SULFAMERAZINE	-2.79
SULFAMETER	29.00
SULFAMETHAZINE	6.45
SULFAMETHIZOLE	-3.22
SULFAMETHOXAZOLE	9.14
SULFAMETHOXYPYRIDAZINE	-9.48
SULFAMONOMETHOXINE	-14.08
SULFANILATE ZINC	-3.76
SULFANITRAN	-11.78
SULFAPHENAZOLE	-14.04
SULFAPYRIDINE	17.68
SULFAQUINOXALINE SODIUM	11.09
SULFASALAZINE	13.38
SULFATHIAZOLE	-16.98
SULFINPYRAZONE	-4.15
SULFISOXAZOLE	13.71
SULFISOXAZOLE ACETYL	-225.21
SULINDAC	-76.58
SULISOBENZONE	9.32
SULMAZOLE	-13.80
SULOCTIDIL	-34.47
SULPIRIDE	4.67
SUMATRIPTAN SUCCINATE	-14.25
SUPROFEN	10.52
SURAMIN HEXASODIUM	12.07
SUXIBUZONE	-5.75
SYMCLOSENE	-6.03
SYRINGIC ACID	-22.72
TACRINE HYDROCHLORIDE	-4.51
TACROLIMUS	-39.24

Table A.3 Continued.

TADALAFIL	16.25
TAMOXIFEN CITRATE	-52.87
TANDUTINIB	-9.88
TANGERITIN	-38.20
TANNIC ACID	43.89
TANSHINONE IIA	-218.61
TANSHINONE IIA SULFONATE SODIUM	-49.88
TAPENTADOL HYDROCHLORIDE	0.01
TAURINE	-15.62
TAZOBACTAM	6.96
TEGASEROD MALEATE	28.44
TEICOPLANIN [A(2-1) shown]	-17.53
TELENZEPINE HYDROCHLORIDE	-4.52
TELITHROMYCIN	2.35
TELMISARTAN	-7.79
TEMEFOS	4.40
TEMOZOLAMIDE	-16.37
TENATOPRAZOLE	2.36
TENIPOSIDE	-52.24
TENOXICAM	12.19
TENYLIDONE	-143.05
TERAZOSIN HYDROCHLORIDE	-0.69
TERBINAFINE HYDROCHLORIDE	-1117.66
TERBUTALINE HEMISULFATE	4.13
TERCONAZOLE	-60.28
TERFENADINE	17.60
TERPENE HYDRATE	12.12
TESTOSTERONE	-53.81
TESTOSTERONE PROPIONATE	-167.68
TETRACAINE HYDROCHLORIDE	7.37
TETRACHLOROISOPHTHALONITRILE	38.66
TETRACYCLINE HYDROCHLORIDE	29.71
TETRAHYDROGAMBOGIC ACID	-155.12
TETRAHYDROSAPPANONE A TRIMETHYL ETHER	-48.52
TETRAHYDROZOLINE HYDROCHLORIDE	-8.03
TETRAMIZOLE HYDROCHLORIDE	-26.24
TETRANDRINE	-44.94

Table A.3 Continued.

TETROQUINONE	-32.56
THALIDOMIDE	-13.90
THEAFLAVIN	18.62
THEANINE	-1.99
THEOBROMINE	-1.67
THEOPHYLLINE	3.01
THERMOPSINE PERCHLORATE	22.68
THIABENDAZOLE	-15.55
THIAMINE	-19.66
THIAMPHENICOL	-7.46
THIAMYLAL SODIUM	14.75
THIMEROSAL	75.93
THIOCTIC ACID	15.77
THIODIGLYCOL	6.22
THIOGUANINE	27.41
THIOGUANOSINE	2.62
THIOPENTAL SODIUM	1.27
THIORIDAZINE HYDROCHLORIDE	44.31
THIOSTREPTON	-3.36
THIOTEPA	19.60
THIOTHIXENE	36.75
THIRAM	16.35
THONZONIUM BROMIDE	33.87
THONZYLAMINE HYDROCHLORIDE	1.04
THYMOPENTIN	-31.48
THYMOQUINONE	104.76
TIAPRIDE HYDROCHLORIDE	21.34
TIBOLONE	-67.78
TICARCILLIN DISODIUM	-34.80
TICLOPIDINE HYDROCHLORIDE	-33.35
TIGOGENIN	-0.25
TILARGININE HYDROCHLORIDE	-12.82
TILETAMINE HYDROCHLORIDE	14.75
TILMICOSIN	3.13
TILORONE	7.93
TIMOLOL MALEATE	9.44
TIMONACIC	10.84

Table A.3 Continued.

TINIDAZOLE	-22.68
TIOCONAZOLE	-97.63
TIOPRONIN	-41.87
TIOXOLONE	-177.79
TIRATRICOL	-69.75
TOBRAMYCIN	38.60
TOCAINIDE HYDROCHLORIDE	-37.18
TODRALAZINE HYDROCHLORIDE	5.59
TOLAZAMIDE	-9.24
TOLAZOLINE HYDROCHLORIDE	-19.29
TOLBUTAMIDE	30.92
TOLFENAMIC ACID	-212.92
TOLMETIN SODIUM	6.84
TOLNAFTATE	-49.79
TOLONIUM CHLORIDE	110.45
TOLPERISONE HYDROCHLORIDE	9.07
TOLTRAZURIL	-25.31
TOMATIDINE HYDROCHLORIDE	-28.77
TOMATINE	102.87
TOPIRAMATE	3.41
TOPOTECAN HYDROCHLORIDE	60.75
TOREMIPHENE CITRATE	-44.59
TOTAROL	-862.37
TOTAROL ACETATE	-826.37
TOTAROL-19-CARBOXYLIC ACID, METHYL ESTER	-755.34
TRACAZOLATE	-91.43
TRAMADOL HYDROCHLORIDE	4.34
TRAMIPROSATE	3.11
TRANDOLAPRIL	12.84
TRANEXAMIC ACID	-14.25
TRANILAST	54.21
TRANYLCYPROMINE SULFATE	5.58
TRAVOPROST	-17.24
TRAZODONE HYDROCHLORIDE	5.37
TRETINOIN	-117.23
TRIACETIN	16.51
TRIACETYLRESVERATROL	-109.70

Table A.3 Continued.

TRIAMCINOLONE	-19.98
TRIAMCINOLONE ACETONIDE	14.80
TRIAMCINOLONE DIACETATE	10.70
TRIAMTERENE	14.47
TRICHLORFON	1.40
TRICHLORMETHIAZIDE	-4.65
TRICHLORMETHINE HYDROCHLORIDE	-18.90
TRICLABENDAZOLE	-40.18
TRICLOSAN	-461.75
TRIENTINE HYDROCHLORIDE	-7.01
TRIFLUOPERAZINE HYDROCHLORIDE	-15.55
TRIFLUPROMAZINE HYDROCHLORIDE	22.31
TRIFLURIDINE	-0.28
TRIGONELLINE	18.26
TRIHEXYPHENIDYL HYDROCHLORIDE	12.87
TRILOSTANE	-108.87
TRIMEBUTINE MALEATE	-62.49
TRIMEDLURE	-112.71
TRIMEPRAZINE TARTRATE	-13.39
TRIMETAZIDINE DIHYDROCHLORIDE	-9.75
TRIMETHADIONE	3.91
TRIMETHOBENZAMIDE HYDROCHLORIDE	-30.30
TRIMETHOPRIM	15.17
TRIMETHYLCOLCHICINIC ACID	6.17
TRIMETOZINE	6.72
TRIMIPRAMINE MALEATE	-23.58
TRIOXSALEN	-75.94
TRIPELENNAMINE CITRATE	-5.38
TRIPROLIDINE HYDROCHLORIDE	11.29
TRIPTOPHENOLIDE	-109.10
TRISODIUM ETHYLENEDIAMINE TETRACETATE	20.22
TROCLOSENE POTASSIUM	-5.52
TROLOX	-9.17
TROPICAMIDE	-23.32
TROPISETRON HYDROCHLORIDE	19.03
TROXERUTIN	6.28
TRYPTAMINE	-25.58

Table A.3 Continued.

TRYPTOPHAN	7.37
TUAMINOHEPTANE SULFATE	5.64
TUBOCURARINE CHLORIDE	15.01
TULOBUTEROL HYDROCHLORIDE	-5.97
TYLOSIN TARTRATE	-15.21
TYLOXAPOL	-58.16
TYRAMINE	-10.99
TYROTHRICIN	84.13
UBIDECARENEONE	2.84
UMBELLIFERONE	7.70
UNDECYLENIC ACID	-15.54
URACIL	3.14
URAPIDIL HYDROCHLORIDE	-21.20
UREA	-0.95
URETHANE	5.65
URIDINE	-7.92
URIDINE TRIPHOSPHATE TRISODIUM	-24.68
URSINOIC ACID	3.61
URSOCHOLANIC ACID	-149.90
URSODIOL	-4.00
USNIC ACID	-32.33
UTILIN	-35.60
VALACYCLOVIR HYDROCHLORIDE	16.30
VALERYL SALYCILATE	-46.14
VALGANCICLOVIR HYDROCHLORIDE	11.73
VALINOMYCIN	-225.04
VALPROATE SODIUM	13.92
VALSARTAN	-19.71
VANCOMYCIN HYDROCHLORIDE	12.52
VARDENAFIL HYDROCHLORIDE	-15.73
VECURONIUM BROMIDE	4.67
VENLAFAXINE HYDROCHLORIDE	12.69
VERAPAMIL HYDROCHLORIDE	-4.96
VERATRIC ACID	5.05
VERATRINE SULFATE	8.90
VESAMICOL HYDROCHLORIDE	-10.78
VIDARABINE	-19.46

Table A.3 Continued.

VINBLASTINE SULFATE	-32.74
VINCRISTINE SULFATE	-21.89
VINORELBINE	-71.32
VINPOCETINE	-2.93
VIOMYCIN SULFATE	28.84
VISNAGIN	-8.12
VORICONAZOLE	-45.42
VORINOSTAT	-153.07
VULPINIC ACID	-3.20
WARFARIN	-13.64
XANTHONE	-100.74
XANTHOPTERIN	24.22
XANTHOXYLIN	-7.97
XANTHURENIC ACID	1.43
XANTHYLETIN	-82.39
XYLAZINE	-0.47
XYLOCARPUS A	-31.38
XYLOMETAZOLINE HYDROCHLORIDE	-28.90
XYLOSE	12.29
YOHIMBIC ACID HYDRATE	-6.79
YOHIMBINE HYDROCHLORIDE	-10.06
ZALCITABINE	0.01
ZALEPLON	33.32
ZAPRINAST	6.34
ZIDOVUDINE [AZT]	-5.33
ZILEUTON	-51.13
ZINC UNDECYLENATE	-14.47
ZIPRASIDONE MESYLATE	-291.85
ZOLMITRIPTAN	18.79
ZOMEPIRAC SODIUM	-10.41
ZOPICLONE	41.07
ZOXAZOLAMINE	-61.79

Several compounds that were identified in the small molecule screen of the Spectrum Collection (6944-0074 and G856-6223 are structural analogues of robustic acid and were purchased from ChemDiv, San Diego, CA) for inhibitors of AC2 activity in HEK-hAC2 cells were further characterized for the ability to modulate cAMP accumulation in response to activation of AC isoforms in HEK-hAC2 (activation by 50 nM PMA, 3 μM forskolin, and 300 nM PGE2), HEK-hAC1 (activation by 3 μM A23187), HEK-hAC5 (activation by 300 nM forskolin), and HEK-wt cells (activation by 3 μM forskolin) as described in chapter 4 (Table A.4).

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Table A.4 AC isoform-selectivity profiles of test compounds in intact-cell studies. AC isoform selectivity was assessed by testing the ability of test compounds (30 µM) to modulate cAMP responses in HEK-hAC2 cells, HEK-hAC1 cells, HEK-hAC5 cells, and HEK-wt cells. Data are reported as a percent of the vehicle treatment condition and represent the Mean ± S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ compared to vehicle condition, one sample *t*-test compared to 100.

	AC2			**AC1**	**AC5**	**WT**
	50 nM PMA	**3 µM FSK**	**300 nM PGE2**	**3 µM A23187**	**300 nM FSK**	**3 µM FSK**
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Amlodipine Besylate	66±13	90±3.0	75±2.8*	140±14	110±4.8	110±15
Nifedipine	180±22	160±8.3*	260±14**	110±16	130±11	150±11*
Nicardipine HCl	270±48	110±9.0	230±28*	150±24	110±2.0*	120±13
6944-0074	220±27*	100±8.7	210±13*	130±9.5	120±6.3	170±11*
G856-6223	200±51	110±3.9	170±10*	120±8.2	110±11	160±7.4*
Fluphenazine diHCl	78±12	180±18	93±7.5	62±5.0*	84±6.4	99±5.2
Methiothepin Maleate	69±11	220±2.0***	76±8.1	45±1.8**	96±7.0	120±8.1
Perphenazine	65±6.1*	170±13*	81±8.5	59±5.6*	93±2.3	110±4.9
(-)-Riboflavin	88±24	64±27	86±29	76±8.3	150±40	260±50
Deoxyadenosine	64±4.2*	93±7.1	100±5.1	94±6.3	94±4.1	85±5.2
BisI	2.3±1.4***	88±3.5	90±9.3	81±4.3*	95±4.9	100±1.5

VITA

VITA

Jason Michael Conley was born on December 12, 1983 to Rick and Mary Jo Conley. Upon graduation from Blackhawk High School in 2002, he attended Washington & Jefferson College where he graduated *cum laude* with a Bachelor of Arts degree in biochemistry. While a student at Washington & Jefferson College, he was introduced to scientific research in a bioorganic research methods intersession class. The following summer, he was awarded a National Science Foundation-Research Experience for Undergraduates fellowship and advanced his organic chemistry research experience at The Ohio State University in the lab of Dr. Leo A. Paquette. His biochemistry background and the positive experiences in organic chemistry research stimulated his aspirations to pursue a career in scientific research. Jason planned to expand his scientific education and was admitted to the Medicinal Chemistry & Molecular Pharmacology graduate program at Purdue University in 2006. Prior to beginning graduate school, he sought to complement his organic chemistry background with a biological science research experience. Thus, he moved to Purdue University three months before the start of graduate school and studied dopamine receptor pharmacology in the laboratory of Dr. Val J. Watts. He

became fascinated by the pharmacology research in the Watts laboratory and consequently studied the molecular aspects of adenylyl cyclase signaling for his dissertation research under the direction of Dr. Watts.