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Small-molecule modulators of inward rectifier K+ channels: recent advances and future possibilities

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Abstract

Inward rectifier potassium (Kir) channels have been postulated as therapeutic targets for several common disorders including hypertension, cardiac arrhythmias and pain. With few exceptions, however, the small-molecule pharmacology of this family is limited to nonselective cardiovascular and neurologic drugs with off-target activity toward inward rectifiers. Consequently, the actual therapeutic potential and 'drugability' of most Kir channels has not yet been determined experimentally. The purpose of this review is to provide a comprehensive summary of publicly disclosed Kir channel small-molecule modulators and highlight recent targeted drug-discovery efforts toward Kir1.1 and Kir2.1. The review concludes with a brief speculation on how the field of Kir channel pharmacology will develop over the coming years and a discussion of the increasingly important role academic laboratories will play in this progress.

Members of the inward rectifier family of potassium (Kir) channels regulate a myriad of physiological processes, including cardiac function, pain processing and opioid action, learning and memory, insulin secretion and epithelial solute transport [1,2]. Some inward rectifiers occupy unique physiological niches that raise intriguing questions about their potential as therapeutic targets. Unfortunately, however, the small-molecule pharmacology of inward rectifiers has remained essentially undeveloped since the first member was cloned nearly 20 years ago [3]. This dearth of pharmacological tools has hindered efforts to develop even a cursory understanding of the physiology of some Kir channels and represents a critical barrier to defining their therapeutic potential. The main goals of this review article are:

- To provide a comprehensive summary of disclosed small-molecule modulators of Kir channels, highlighting the few examples where pharmacology has illuminated a deeper understanding of their physiology and 'druggability';

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- To review recent advances and future possibilities in targeted drug-discovery efforts directed at Kir channels.

Overview of Kir channel structure & function

The term 'rectification' refers to a nonlinear change in ionic current through an ion channel pore as a function of the electrochemical driving force. By convention, the movement of a cation from the extracellular solution to the cytosol is defined as an inward current. Thus, Kir channels preferentially conduct K^+ ions inwardly under voltage-clamp conditions [1,2].

Inward rectification is caused by blockade of the channel pore by intracellular cations such as magnesium and polyamines (e.g., spermine, putrescine) driven 'outwardly' by membrane depolarization. The extent of pore block and, hence, strength of rectification varies widely among different family members. Strong rectifiers pass very little outward current, whereas weak rectifiers do so across a broad range of potentials [4,5]. In general, strong rectifiers are expressed in excitable cells, such as cardiac myocytes or neurons, where they tend to hyper polarize the membrane potential, but avoid short-circuiting action potentials by limiting outward K^+ current during depolarization. In contrast, weak rectifiers carry significant outward current and are, therefore, well suited to function in nonexcitable tissues, such as secretory epithelia [1].

The recent determinations of high-resolution x-ray structures of Kir channel proteins have significantly advanced our molecular understanding of inward rectification [6–10]. They also create unique opportunities for understanding small-molecule–Kir channel interactions with near atomic-level resolution. To facilitate the present discussion, we include a brief overview of the relevant structural elements implicated in small-molecule binding.

Kir channels are tetramers comprised of four identical (homotetrameric) or homologous (heterotetrameric) membrane-spanning subunits surrounding a water-filled pore through which K^+ ions move down their electrochemical gradient. FIGURE 1 shows a homology model of the Kir1.1 cytoplasmic domain docked to the membrane-spanning portion of a Kir3.1–KirBac1.3 chimera [11]. Two subunits have been removed for clarity. Each channel subunit consists of two membrane-spanning α-helical domains (TM1 and TM2) separated by an extracellular loop that forms the narrow K^+-selectivity filter (SF). TM2 lines the membrane-spanning pore and terminates near the membrane–cytoplasm interface in a structure termed the helix bundle crossing (HBC). Structural and mutagenesis studies suggest that the HBC functions as a regulatable gate that opens and closes in response to diverse cell-signaling molecules, such as extracellular K^+, intracellular protons and phosphoinositides [12]. The narrow gating-loop positioned near the HBC may also function as a gate in series with the HBC [7]. The extensive cytoplasmic domain extends the conduction pore well beyond the membrane and into the cytosol [6–10].

Crystal structure-guided mutagenesis studies have identified three 'rings' of negatively charged residues that participate in rectification. These are highlighted in FIGURE 1 and labeled RC (rectification controller), rectification modulator 1 (RM1) and rectification modulator 2 (RM2). Electrostatic interactions between these negatively charged rings and positively charged polyamines produce high-affinity binding and block in strong rectifiers. Weak rectifiers possess uncharged amino acids at one or more of these positions, thereby reducing the polyamine block [5,7,10]. Importantly for this review, an emerging body of evidence suggests that the unique charge distribution and architectural topography near RC, RM1 and RM2 may provide selective binding pockets that can be targeted with small-molecule inhibitors [13,14]. These will be discussed further below.

Kir channel pharmacology & therapeutic potential

Kir1.1 (ROMK)

Kir1.1, which is commonly referred to as the renal outer medullary K^+ (ROMK) channel, is the founding member of the inward rectifier family. The Kir1.1 cDNA was cloned from rat kidney outer medulla and was shown to encode a weakly rectifying K^+ channel with functional properties very similar to those of the apical membrane small-conductance secretory K^+ (SK) channel expressed in epithelial cells of the thick ascending limb (TAL) and collecting duct (CD) [3]. The SK channel is absent from the TAL and CD of mice carrying a deletion in the gene encoding Kir1.1 *(KCNJ1)*, establishing that the SK current is carried by Kir1.1 [15].

The significance of Kir1.1 in renal tubule function was appreciated more than a decade before the channel was cloned. The major functions of Kir1.1 in the nephron are summarized in FIGURE 2. In the TAL, luminal K^+ recycling by Kir1.1 catalyzes NaCl reabsorption by the Na^+-K^+-$2Cl^-$ co-transporter and **loop diuretic** target NKCC2, which in turn promotes osmotic water reabsorption in the distal nephron [16–18]. In the connecting tubule and CD, Kir1.1 constitutes a key physiological pathway for regulating K^+ secretion to match dietary intake [19,20].

The identification of disease-causing mutations in *KCNJ1* in patients with Type II Bartter syndrome (BS) [21] established the importance of Kir1.1 in kidney function in humans. BS is a life-threatening renal 'tubulopathy' characterized by excessive urination, renal Na^+ and K^+ wasting, normal to low blood pressure and metabolic alkalosis. BS is actually a group of rare salt-wasting tubulopathies caused by heritable mutations in at least six genes whose products participate in salt transport in the kidney (for a recent review see elsewhere [22]). Nearly 40 loss-of-function BS mutations in *KCNJ1* have been identified to date. Several of these decrease the open-state probability of Kir1.1, while others impair its trafficking to the cell surface [11, 23–25].

The clinical presentation of Type II BS can be rationalized in terms of diminished luminal K^+ secretory activity in the TAL. As illustrated in FIGURE 2, NKCC2 is functionally coupled to Kir1.1 such that loss of channel activity impairs NaCl reabsorption in the TAL. This has several deleterious consequences. First, Na^+ that would normally be reabsorbed in the TAL is delivered to the distal nephron and ultimately lost in the urine. Second, the ensuing dilution of the medullary interstitium blunts osmotic water reabsorption in the CD and thereby increases urine output. Third, increased delivery of fluid to the distal nephron stimulates K^+ secretion [26] and urinary K^+ loss and thereby reduces plasma K^+ levels (**hypokalemia**). This, in turn, promotes urinary acid excretion and metabolic alkalosis [27].

As shown in FIGURE 2, K^+ secretion in the distal nephron is mediated by two K^+-conductive pathways in the apical plasma membrane. Under 'normal' conditions, Kir1.1 is thought to provide the major route for K^+ efflux. However, an emerging body of evidence suggests that during periods of high tubule flow, such as those experienced during diuresis, 'flow-stimulated' large-conductance K^+ (BK or maxi-K) channels take on a more prominent role [28–30]. Perhaps not surprisingly, the severity of hypokalemia observed in Type II BS is generally milder than that of Type I BS [21], a related tubulopathy caused by mutations in the loop diuretic target NKCC2 [22,31,32]. One simple explanation is that the K^+-secretory capacity in Type I patients is greater due to the expression of both Kir1.1 and BK channels in the distal nephron and is lower in Type II patients due to the absence of Kir1.1. Studies in Kir1.1-knockout mice, a genetic model of Type II BS, showing a reduction in distal nephron K^+ secretion support this notion [33]. The loss of Kir1.1 in mice appears to be compensated for by an increase in BK channel expression [33]. Upregulation of flow-stimulated BK channels

in the setting of chronic diuresis and, thus, high tubule flow may account for the relatively mild K^+ wasting seen in Type II BS patients.

Type II BS is an autosomal-recessive disorder requiring the inheritance of two mutant *KCNJ1* alleles for expression of the disease. Interestingly, Lifton and colleagues recently identified within the Framingham Heart Study cohort heterozygous carriers of *KCNJ1* mutations who do not exhibit the salt- and water-wasting phenotype of BS [34]. Importantly, however, these individuals have lower blood pressure and a reduced prevalence of hypertension. Similarly, Tobin *et al*. identified in the general population single nucleotide polymorphisms in noncoding regions of *KCNJ1* that are associated with lower blood pressure. No associations with Na^+ or K^+ excretion were found [35]. Taken together, these observations suggest that a partial loss of Kir1.1 function may reduce blood volume and pressure without causing pathological derangement of electrolyte balance observed in BS. They also raise the intriguing possibility that Kir1.1 is a molecular target for a novel class of loop diuretic.

Conventional loop diuretics (e.g., flurosemide, bumetanide and torasemide) increase urine production by inhibiting NKCC2 activity in the TAL (FIGURE 2) [36]. The drug response is virtually identical to the clinical presentation of Type I BS (see previous discussion), including potentially life-threatening reductions in serum K^+ [37,38]. In light of the preceding discussion, it is conceivable that small-molecule antagonists of Kir1.1 could trigger diuresis by acting at the TAL, but do so with minimal K^+ wasting by inhibiting K^+ secretion in the distal nephron (FIGURE 2). However, assessing the therapeutic potential of Kir1.1 will first require the development for selective drug-like probes for *in vivo* pharmacology experiments.

In an effort to overcome this barrier, Denton and colleagues set out to develop a high-throughput screen (HTS) for small-molecule modulators of Kir1.1 function. The chosen assay measures inwardly directed movement of the K^+ congener thallium (Tl^+) through Kir1.1 channels using a Tl^+-sensitive fluorescent dye. Similar assays have been developed for KCNQ2, KCa2.1, KCa2.2, KCa2.3 [39–41], and hERG [42] K^+ channels and the K^+-Cl^- co-transporter KCC2 [43].

A stable HEK-293 cell line expressing Kir1.1 and under the control of a tetracycline (Tet)-inducible promoter was employed to avoid cytotoxic effects of constitutive Kir1.1 expression [23,44] and provide a convenient internal control for screens. The Kir1.1-S44D mutant, in which serine (S) at position 44 was mutated to aspartate (D), was used as a surrogate for the wild-type channel in an effort to maximize cell surface expression of the channel [45,46]. One clone exhibiting robust Tet-inducible Tl^+ flux, Kir1.1 channel activity (FIGURE 3A & B) and biochemical localization to the cell surface was chosen for assay development and HTS [47].

FIGURE 3B shows representative fluorescence traces recorded from individual wells of a 384-well plate containing uninduced (−Tet) or Tet-induced cells bathed in control (+Tet) or Tertiapin-Q (TPNQ)-containing assay buffer (Tet + TPNQ). Extracellular Tl^+ addition evoked an abrupt increase in fluorescence in Tet-induced cells, but not in uninduced cells or induced cells pretreated with the Kir1.1 inhibitory peptide TPNQ. Using this assay, approximately 225,000 small molecules were screened in 384-well plates at a single dose of 10 μM.

FIGURE 4A shows the structure of one Kir1.1 inhibitor, termed VU590, identified by HTS. In Tl^+ flux assays, VU590 inhibited Kir1.1 in a dose-dependent manner with an IC_{50} of approximately 300 nM (FIGURE 4B). In whole-cell patch clamp electrophysiological experiments on transiently transfected HEK-293 cells, VU590 inhibited wild-type Kir1.1 in a dose-dependent manner, but had no effect on either Kir2.1 or Kir4.1 at a concentration of 10 μM. Importantly, however, VU590 at this concentration inhibited Kir7.1 by approximately 65% (FIGURE 5). This was a serendipitous finding because the only other inhibitors of this newest and perhaps least understood family member are the nonselective, millimolar

concentration pore blockers Cs^+ and Ba^{2+} [48]. Under the appropriate experimental conditions, VU590 should be a useful tool for sorting out the functions of Kir7.1 in numerous cell types [48–51].

Medicinal chemistry efforts around the VU590 scaffold were initiated with the goal of improving its potency and defining the VU590 moieties required for high-affinity Kir1.1 activity. VU590 structure–activity relationships proved shallow, however, yielding only analogs with IC_{50} values in the 5–100 μM or greater (inactive) range of concentrations. Moving the nitro group from the 4-position in VU590 to the 3- or 2-position or to a 2,4-dinitro analog led to partial or complete loss of potency. Alternate functional groups for the 4-nitro moiety, such as 4-Cl, 4-thiomethyl and 4-methyl ester, also afforded weak inhibitors. All attempts to attenuate the basicity of VU590 by replacing the tertiary amine moieties with amides, sulfonamides and ureas resulted in inactive compounds (FIGURE 6). Thus, VU590 is a unique Kir1.1 inhibitor with a particular balance of physiochemical properties [47].

As a first step toward defining the VU590 binding site, a series of electrophysiological experiments were performed to define the 'sidedness' of VU590 action on Kir1.1. It was noted that the rate of VU590 inhibition was rather slow, requiring up to 2 min for full block, and washout was incomplete. This suggested that VU590 might first cross the cell membrane to reach an intracellular binding site. It was also apparent that the membrane potential at which Kir1.1 currents rectify became increasingly negative during the onset of channel block. As discussed earlier, rectification is a result of block of outward current by intracellular cations [4,52–54]. The hyperpolarizing shift in rectification potential was therefore interpreted as another indicator of an intracellular binding site [47]. In another series of experiments, it was shown that VU590 could be displaced from the pore by inwardly directed K^+ ions [47]. This phenomenon, known as '**knok-off**' [55], has been reported for the intracellular Kir channel pore blockers chloroquine [13] and nortriptyline [14] (TABLE 1). Taken together, these data support a model in which VU590 crosses the membrane to access an intracellular binding site within the Kir1.1 channel pore. Mutagenesis, electrophysiology and molecular modeling are being used to map the VU590 binding site in Kir1.1 and Kir7.1. These studies will shed light on which structural properties of the channel and molecule confer moderate selectivity to VU590.

Kir2

The Kir2 family members 2.1–2.4 are strong rectifiers expressed primarily in heart, brain and skeletal muscle, with lesser expression in the kidney and gut [1,2]. Dominant negative mutations in Kir2.1 underlie Andersen–Tawil syndrome characterized by periodic paralysis, cardiac ventricular **arrhythmias** related to prolonged QT interval and dysmorphic facial features [56]. Thus, Kir2.1 is an unlikely candidate for drug-development efforts. However, some have suggested that Kir2.3 antagonists may be useful for certain cardiac arrhythmias. Unlike Kir2.1, which is expressed in both ventricular and atrial myocardium, Kir2.3 is primarily expressed in atrial tissue leading some to postulate that selective Kir2.3 antagonists may be useful for the treatment of atrial fibrillation (discussed in the following section) [57].

Levitan and colleagues performed a high-throughput screen for small-molecule modulators of Kir2.1 heterologously expressed in yeast [58]. Their assay exploits the ability of Kir2.1 (as well as other K^+ channels) to rescue a yeast strain deficient in two K^+-transport proteins, TRK1 and TRK2, when grown under low-K^+ conditions that would otherwise kill the yeast. In a screen of 10,000 small molecules, they identified several compounds that inhibited Kir2.1-dependent yeast growth. The first published compound, termed 3-bicyclo[2.2.1] hept-2-yl-benzene-1,2-diol (TABLE 1), inhibits Kir2.1 in a voltage-independent manner with an IC_{50} of approximately 60 μM. Interestingly, however, they found the compound preferentially inhibits the voltage-gated K^+ channel Kv2.1 with much greater potency (IC_{50} = 1 μM). They went on

to show that 3-bicyclo[2.2.1] hept-2-yl-benzene-1,2-diol has neuroprotective effects associated with inhibition of native Kv2.1 channels in neuronal cultures [58]. This study highlights how post-HTS secondary assays can lead to unexpected and important discoveries in ion channel pharmacology.

The second Kir2.1 inhibitor reported from the yeast screen is gambogic acid (GA) [59], a potent inducer of apoptosis that has been considered for anticancer therapy (TABLE 1). During acute applications, GA inhibits Kir2.1 by only 30% and Kv2.1 by 70% at 10 μM. During prolonged exposures (up to 3 h), however, the compound inhibits Kir2.1 with an IC_{50} of 27 nM, an effect that was not observed in Kv2.1, Kir1.1 or hERG K^+ channels. They further showed that 1-μM GA causes Kir2.1 and Kv2.1 partitioning into biochemically distinct (i.e., Triton X-100-soluble) plasma membrane microdomains, suggesting that perturbation of channel interactions with regulatory proteins and/or lipids that are specific to Kir2.1 accounts for the GA-induced inhibition of channel activity [59].

Min Li and co-workers developed another assay that uses atomic absorption spectrophotometry to monitor recovery of Kir2.1 channel activity after chemically inactivating the channels present in the plasma membrane [60]. Using this Functional Recovery After Chemobleaching (FRAC) assay, they screened 2000 small molecules for compounds that altered the time course of recovery [61]. The neuroprotective compound celastrol was found to reduce cell-surface expression of Kir2.1 at sub-micromolar (e.g., 200-nM) concentrations and channel activity at low micromolar (e.g. 20-μM) concentrations. Celastrol had similar effects on hERG (TABLE 1) [61].

A handful of other low-affinity inhibitors of Kir2 channels have been identified in studies motivated by the intent of implicating Kir2 antagonism in drug intoxication-related cardiac or neurologic side effects. For example, the arrhythmogenic effects of chloroethylclonidine and the first-generation anti-histamines mepyramine and diphenhydramine (TABLE 1) have been attributed to low-affinity Kir2.1 antagonism [62,63]. Ventricular arrhythmias may also occur with toxic levels of the antimalarial agent chloroquine. Chloroquine inhibits Kir2.1 with an IC_{50} of approximately 10 μM (TABLE 1). Electrophysiological analysis revealed that chloroquine blocks Kir2.1 in a voltage-dependent fashion and exhibits knock-off with inwardly directed K^+ current, suggesting a binding site located in the cytoplasmic pore. Site-directed mutagenesis and molecular docking simulations indicated that chloroquine binds to a superficial location in the cytoplasmic pore through electrostatic interactions with the negatively charged residues E224, D259, E299 and D255 near RM1 and RM2 (FIGURE 1), with a minor contribution from hydrophobic interactions with a nearby phenylalanine residue [13]. These data suggest that the cytoplasmic pore region around RM1 and RM2 may potentially represent a targetable small-molecule binding site. The estrogen receptor antagonist tamoxifen (TABLE 1) blocks Kir2 channels with slightly greater potency for Kir2.3 (IC_{50} 0.31 μM) over Kir2.1 and Kir2.2 (IC_{50} 0.93 and 0.87 μM) and is thought to interfere with the channel PIP_2 interaction. Tamoxifen's preferential inhibition of Kir2.3 parallels its slight preference for atrial over ventricular inward rectifier current suggesting that selective Kir2.3 inhibitors may indeed dampen atrial arrhythmias and have little effect on ventricular excitability [64].

Investigators studying the effects of neurosteroids on Kir2 channels unexpectedly found that pregnenolone sulfate (PS) preferentially activates Kir2.3 with a half-maximal concentration of 16 μM and a maximal current potentiation of approximately 80% (TABLE 1). PS appears to be selective in that it has no effects on Kir1.1, Kir2.1, Kir2.2 or Kir3.1 currents. Several structurally related neurosteroids were found to be inactive. Preliminary data suggest that PS acts directly on Kir2.3 at an extracellular site, although the precise binding site location has not yet been defined [65]. Most Kir channel activators act intracellularly to change channel

gating. These data raise the intriguing possibility that extracellularly acting small molecules may also modulate Kir channel gating.

Kir3 (GIRK)

Kir3 channels are distinguished from other inward rectifiers in that their activity is critically regulated by G protein-coupled receptors (GPCRs) [2]. Accordingly, members of this family are often referred to as GPCR-activated inward rectifier K^+ channels (GIRK). These channels exhibit a low open-state probability under basal conditions, but are activated through a Gβγ-dependent pathway following GPCR stimulation [66]. In mammals, the GIRK subfamily is comprised of four members: Kir3.1, Kir3.2, Kir3.3 and Kir3.4. GIRK channels exist primarily as heterotetramers, with Kir3.1/3.2 predominating in the nervous system and Kir3.1/3.4 in the heart [67,68]. While Kir3.1 subunits do not form functional homomeric channels, other homomeric and heteromeric subunit combinations are possible when expressed in heterologous systems. Except for the prominent role of Kir3.2/3.3 heteromers in midbrain dopaminergic signaling, the physiological significance of other subunit combinations remains unclear [68–71]. In general, Kir3 channels activated by GPCR signaling pathways dampen excitability broadly throughout the nervous system and in the heart. GIRK channels are thus uniquely poised to function as drug targets for disorders of excessive cell excitability, such as cardiac arrhythmias, seizure disorders and pain. However, the limited pharmacology of the GIRK subfamily has thus far hampered efforts to fully evaluate their actual therapeutic potential.

Kir3.1/3.4 heteromeric channels are expressed primarily in atrial myocytes of the heart where they are functionally coupled to M2 muscarinic GPCRs. **Acetylcholine** released onto M2 receptors following vagal nerve stimulation induces an outward K^+ current through Kir3.1/3.4 channels, which hyperpolarizes the cell membrane potential. The classical term for this acetylcholine-activated inward rectifier K^+ current is I_{KACh} [69]. Activation of I_{KACh} is thought to have several important effects on cardiac function, including slowing of the heart rate and shortening of the atrial myocyte action potential and effective refractory period [72].

Studies of Kir3 knockout mice have raised intriguing questions about the therapeutic potential of cardiac GIRK for the treatment of atrial fibrillation, the most common cardiac dysrhythmia encountered in clinical practice [73]. Knockout of Kir3.1 or Kir3.4 abolishes I_{KACh}, confirming their role in acetylcholine modulation of cardiac function [74,75]. Kir3.4 knockout mice exhibit unchanged ambulatory heart rate, reduced heart rate variability and resistance to atrial fibrillation [72,75]. These and other data (discussed below) suggest that small-molecule antagonists of cardiac GIRK may act as anti-arrhythmic agents with little effect on resting cardiac function.

Several anti-arrhythmics are limited by adverse off-target effects on ventricular ion channels and excitability with resultant ventricular arrhythmias or by noncardiac side effects. A recent focus has been the development of agents that selectively target atrial excitability. Cardiac GIRK expression is largely restricted to the atria, suggesting it may be a suitable target for drug development [57]. A growing body of evidence lends support to this notion. For example, currently prescribed medications, such as amiodarone, as well as several drugs in development for atrial fibrillation (vernakalant, dronedarone, AVE0118, JTV-519) are known inhibitors of I_{KACh} (TABLE 1). Vernakalant has reached clinical trials and shown efficacy in terminating recent onset atrial fibrillation [76]. However, vernakalant, AVE0118, and JTV-519 also inhibit Kv1.5 or Kv4 channels expressed in the heart [76–79]. It is therefore difficult to conclude from studies with these multi-channel blockers whether selective GIRK antagonists will be therapeutically beneficial.

Nissan Chemical Industries has developed several benzopyrane derivatives in an attempt to target cardiac GIRK selectively. One of their first disclosed compounds, NIP-142 (TABLE 1),

inhibits Kir3.1/3.4 channels with submicromolar affinity, but also blocks Kv1.5 channels with similar potency. NIP-142 has shown efficacy in terminating vagally mediated atrial fibrillation with mild prolongation of ventricular repolarization [80]. Another derivative, NIP-151, more potently inhibits Kir3.1/3.4 channels with an IC_{50} of 1.6 nM and terminates atrial fibrillation without any effect on ventricular excitability in animal models [81]. Future studies comparing the efficacy of NIP-151 and NIP-142 or vernakalant may be helpful in establishing the therapeutic potential of selective Kir3 inhibitors for atrial fibrillation.

Kir3.1/3.2 heteromeric channels are broadly expressed in the nervous system, where they act as effectors for a myriad of neurotransmitter systems including GABA, opioid, glutamate, adenosine, and dopamine. In general, GIRK channels are found postsynaptically in perisynaptic regions although limited postsynaptic density and presynaptic expression has been reported [68]. GIRK activation leads to membrane hyperpolarization and dampening of postsynaptic excitation [82,83]. GIRK channels fine-tune synaptic transmission underlying memory storage, control of seizure activity, reinforcement of addictive substance use and regulation of pain sensation.

Consistent with their broad distribution and negative feedback on excitatory synaptic transmission, Kir3.1- and Kir3.2-knockout mice exhibit many behavioral phenotypes including hyperactivity, reduced anxiety behavior, diminished administration of addictive substances, lower seizure threshold, hyperalgesia and diminished opioid-induced analgesia [84–86]. By contrast, Kir3.3 knockout mice exhibit only reduced self-administration of cocaine and possible hyperalgesia in certain experimental paradigms consistent with the more limited midbrain expression of Kir3.3 subunits, particularly in the dopaminergic reward pathway [70,87]. In light of these phenotypes, it is difficult to envision centrally acting Kir3.1/3.2 channel modulators providing therapeutic benefit without significant deleterious side effects. However, genetic models are mired with developmental compensatory mechanisms and may not accurately reflect beneficial or adverse responses to acute and graded pharmacologic modulation in genetically normal individuals.

At present, most of the work on neuronal GIRK pharmacology has focused on well-characterized neurologic drugs exhibiting low-affinity activity toward GIRK. For example, the antipsychotic agent thioridazine, the tricyclic antidepressant nortriptyline, the serotonin reuptake inhibitor fluoxetine, the anesthetics bupivacaine and halothane and the anti-epileptic agent ethosuximide have all been shown to inhibit Kir3 channels at micromolar-to-millimolar concentrations (TABLE 1) [88–93]. Interestingly, two nanomolar-affinity GPCR-directed small molecules, SCH23390 (D1 receptor antagonist) and U50488H (κ-opioid receptor agonist) were found to directly inhibit GIRK at low micromolar concentrations [94,95]. Some investigators have proposed that the off-target activity toward GIRK may explain some of the adverse side effects observed during high-dose intoxication events with these agents. To our knowledge, however, no direct role of GIRK antagonism in drug toxicity has been established.

Kir3.1/3.2 activators may be useful in pain management. Opiates remain the most clinically effective analgesics primarily because they activate receptors that dampen synaptic transmission at multiple points along the pain pathway. GIRK channels represent an effector pathway for opiates and therefore theoretically possess the same capability to diffusely diminish pain pathway activity [96]. Furthermore, GIRK activators may not share opiate related side effects such as addiction, tolerance, withdrawal, and constipation [97]. Ultimately, however, a clear understanding of the therapeutic potential of neuronal GIRK channels awaits the development of selective Kir3.1/3.2 channel modulators.

Kir4 & Kir5

Kir4 (Kir 4.1 and 4.2) and the Kir5.1 family members are expressed primarily in CNS and retinal glial cells, inner ear cochlear cells and kidney distal tubular epithelial cells [98–102]. Kir4 subunits can form functional homomeric channels, whereas Kir5.1 must co-assemble with Kir4 to participate in ion channel function [103].

Genetic knockout of Kir4.1 in mice leads to CNS myelination defects, persistent neuronal depolarization secondary to diminished glial cell K^+ uptake, retinal dysfunction and cochlear dysfunction [104–106]. Similarly, patients with a recently discovered genetic syndrome termed EAST or SeSAME syndrome due to loss-of-function mutations in Kir4.1 exhibit seizures, sensorineural deafness, ataxia and mental retardation [107,108]. These findings confirm that Kir4.1 plays a critical role in K^+ homeostasis in the human CNS. Given these adverse phenotypes, the therapeutic value of centrally active Kir4.1 antagonists is doubtful. It is formally possible, however, that Kir4/5 channel activators may promote glial cell K^+ uptake and thereby exhibit some anti-epileptic activity [109].

Interestingly, SeSAME syndrome patients also exhibit renal Na^+ wasting, raising the possibility that Kir4.1 in the nephron may be a target for novel diuretics. In theory, these could offer some advantages over existing diuretics, all of which target transport proteins located on the apical tubular membrane. This requires that a diuretic is filtered or secreted into the urinary filtrate to reach its target. These agents lose potency with diminishing renal function, a common problem with diuretic use in chronic kidney disease and congestive heart failure patients [36]. Antagonists targeting basolaterally expressed Kir4/5 should increase renal salt wasting independent of renal function since their molecular target is located on the blood side of the nephron [101,102]. Of course, these compounds would have to be restricted from crossing the blood–brain barrier to avoid the aforementioned neurologic consequences.

No selective Kir4/5 modulators have thus far been disclosed. However, investigators studying antidepressant drug modulation of glial cell function have demonstrated that many of these drugs inhibit Kir4 with relatively low affinity. Tricyclic antidepressants almost universally block Kir4.1 currents with nortriptyline exhibiting a voltage-dependent IC_{50} ranging from 16–38 μM. The selective serotonin reuptake inhibitor (SSRI) fluoxetine similarly inhibits Kir4.1 with an IC_{50} of 15 μM, but in a voltage-independent fashion. Unlike the tricyclic antidepressants, SSRI activity toward Kir4.1 appears to be restricted to fluoxetine and sertraline. These agents are relatively selective among Kir channels exhibiting minimal inhibition of Kir1.1 and Kir2.1 channels (TABLE 1) [110,111]. However, as noted earlier, other studies have noted that antidepressants also inhibit Kir3 currents although it is difficult to directly compare potency of these agents against Kir3 and Kir4.1 given significant differences in study conditions. Interestingly, the antidepressant activity profile for Kir3 and Kir4.1 channels appears similar with near universal block by tricyclic antidepressants and more restricted SSRI activity centered around fluoxetine [88,110,111]. The common structure–activity relationship between channel families suggests a common Kir channel antidepressant binding site. More recent studies on antidepressant block of Kir4.1 using site-directed mutagenesis and structural modeling have identified a binding site centered around electrostatic interactions involving polar residues (E158 and T128) of the RC region (FIGURE 1) deep within the channel pore [14]. These data highlight a recurring theme in the current state of Kir channel small-molecule pharmacology involving the cytoplasmic entry of antagonists into the channel pore with resultant binding either deep in the pore as with antidepressants and Kir4.1 or superficially as with chloroquine and Kir2.1. Since VU590 block of Kir1.1 currents behaves similarly in electrophysiology experiments (see above), mapping its binding site within the Kir1 and Kir7 channel pore should add to our understanding of these binding sites including the characteristics that engender selectivity and potency.

Kir6

Kir6.1 and Kir6.2 constitute the pore-forming subunits of the adenosine-5'-triphosphate-inhibited K_{ATP} channels that couple cellular metabolism to membrane excitability in several key cell types, including pancreatic β cells, vascular smooth muscle, cardiac sarcolemma and brain. The native K_{ATP} channel complex is comprised of four Kir6 subunits and the same number of sulfonylurea receptor proteins, the latter of which confer channel sensitivity to numerous small-molecule inhibitors and activators. Some of these are currently in use clinically for the treatment of a variety of disorders such as neonatal and adult-onset diabetes, hyperinsulinism, hypertension, cardiac arrhythmia, angina and alopecia. The physiology and molecular pharmacology of the K_{ATP} channel family has been reviewed extensively elsewhere [2,112–114] and will not be discussed further here.

Kir7

Kir7.1 is the newest member of the Kir family and is expressed primarily in brain, intestine, kidney and retina [48]. The recent discovery of Kir7 mutations in patients with Snowflake vitreoretinal degeneration has brought attention to the role of Kir7 in K^+ homeostasis of the **retinal pigmented epithelium** (RPE) [115]. The mixed Kir1.1/7.1 channel blocker VU590 [47] should be useful in understanding the physiology of Kir7.1 in the RPE because, to our knowledge, Kir1.1 is not expressed in those cells.

In polarized epithelial cells of the intestine and nephron, Kir7.1 expression appears to be limited to the basolateral membrane [49,51]. Based on their subcellular co-localization in the gut, it has been postulated that Kir7.1 is functionally coupled to the Na^+-K^+-ATPase and Na^+-K^+-$2Cl^-$ co-transporter and thereby contributes to transepithelial Cl^- secretion [51]. If this model is correct, gut-specific Kir7.1 antagonists may be useful in managing secretory diarrhea [116]. However, to our knowledge, there is currently no evidence to suggest that Kir7.1 channels are functional in the intestine due to their low single-channel conductance [48] and previous lack of pharmacological tools. Because Kir1.1 is not expressed in the intestine, VU590 should help fill in this gap.

Future perspective

An emerging body of physiological and genetic evidence has raised important questions regarding the therapeutic value of inward rectifiers in the treatment of several common cardiovascular and neurological disorders. Despite this potential, the small-molecule pharmacology of the Kir channel family is essentially undeveloped and comprised largely of low-affinity, nonselective cardiovascular and neurologic drugs (TABLE 1). The recent discovery of small-molecule modulators of Kir1.1 in Tl^+ flux-based HTSs [47] is timely and represents what we anticipate to be a new era in targeted drug-discovery efforts directed toward this important channel family. The Tl^+ flux assay should be amenable for screening most inward rectifiers.

We further anticipate that academic laboratories, such as our own, will play an increasingly important role in the development of ion channel probes. Traditionally, this arena has been dominated by the pharmaceutical industry whose primary goal is to develop profitable drugs. Compounds that do not show immediate promise for drug development are often dropped and never disclosed to the public. As discussed throughout this review, there is a pressing need for the discovery of new tools to support basic science efforts to understand the physiology and, in some cases, the druggability of inward rectifiers. We expect that the National Institutes of Health Molecular Libraries Program [201], which funds industry-quality HTS and probe development by academic scientists, will continue to play an indispensible role in these endeavors.

Finally, we anticipate that advances in structural and computational biology will play an important role in developing the small-molecule pharmacology of the inward rectifier family. With the availability of several high-resolution x-ray structures of bacterial and mammalian Kir channel proteins, our structural understanding of inward rectifiers is probably the most advanced of any ion channel family. In this paradigm, small-molecule probes will be used in classical site-directed mutagenesis and electrophysiological experiments to define their molecular binding sites. Computational modeling techniques based on existing x-ray structures and the physicochemical properties of small molecules can then be deployed to explore the molecular interactions in detail. Structure-based hypotheses are then formulated and tested experimentally using mutagenesis, medicinal chemistry and electrophysiology. These multidisciplinary studies will ultimately lead to an atomic-level understanding of selective drug binding sites in Kir channels and enable advanced cheminformatics and virtual screening-based methods of drug discovery.

Executive summary

- Selective Kir channel modulators have several potential therapeutic applications:
  - Kir1.1 antagonists as K^+-sparing diuretics for treatment of hypertension and congestive heart failure
  - Kir2.3 and Kir3.1/3.4 antagonists for treatment of atrial fibrillation
  - Kir3.1/3.2 activators for treatment of pain
  - Kir4.1 peripherally active antagonists as renal function-independent diuretics for the treatment of hypertension
  - Kir7.1 gut-specific antagonists for secretory diarrhea
- Thallium flux-based fluorescence assays have been used to discover novel modulators of Kir1.1 by HTS and should be broadly applicable to other members of the channel family.
- Existing high-resolution x-ray structures should be instrumental in developing the small-molecule pharmacology of the Kir channel family.

KEY TERM

Loop diuretic	Class of diuretic that acts on the loop of Henle of the nephron to inhibit sodium chloride and water reabsorption from the urine
Hypokalemia	Reduction of serum K^+ below 3.5 mEq/l
Knock-off	Voltage-dependent displacement of a blocker from an ion channel pore
Arrhythmias	Abnormal electrical activity in the heart
Acetylcholine	Neurotransmitter that slows the heart rate through activation of GIRK channels
Retinal pigmented epithelium	Pigmented epithelial cell layer that provides essential support functions to the interposed photoreceptors of the eye

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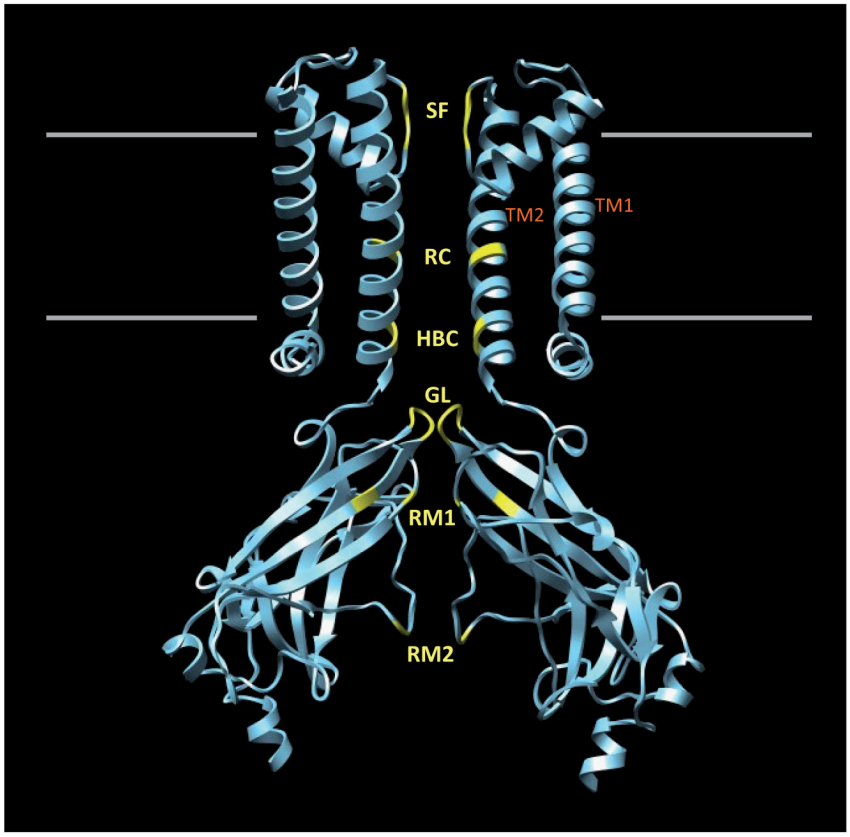

Figure 1. Structural model of an inward rectifier potassium channel

A homology model of the Kir1.1 channel cytoplasmic domain has been docked to the membrane-spanning portion of a Kir3.1–KirBac1.3 channel chimera [11]. Regions of the channel backbone important for channel function, rectification and small-molecule binding are highlighted.

GL: Gating loop; HBC: Helix bundle crossing; RC: Rectification controller; RM: Rectification modulator; SF: Selectivity filter; TM1 and 2: Transmembrane domains 1 and 2.

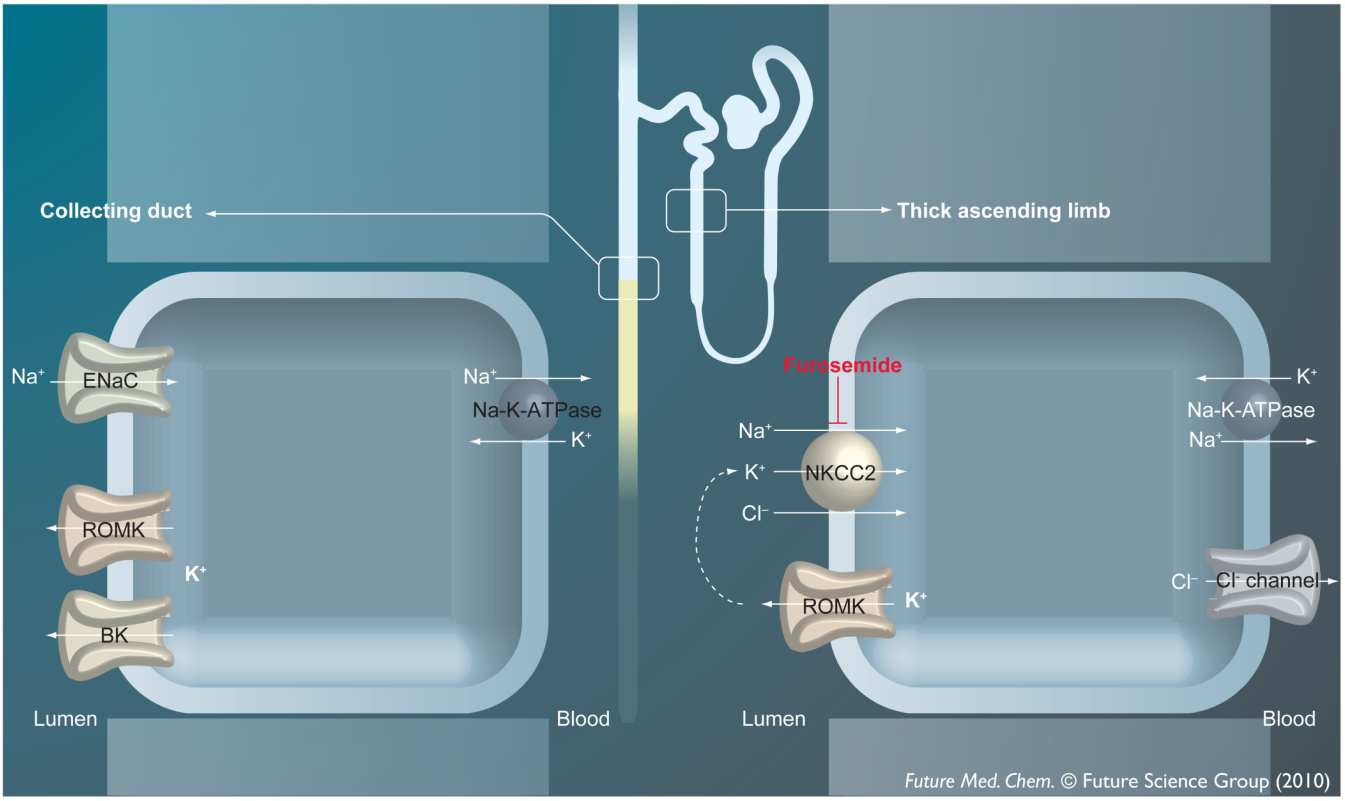

Figure 2. Major functions of ROMK in the kidney tubule

In the thick ascending limb of Henle's loop, ROMK provides substrate K^+ ions essential for transepithelial NaCl reabsorption by NKCC2. NaCl reabsorption generates a hypertonic interstitium that promotes osmotic water reabsorption in the distal nephron. In the collecting duct, ROMK constitutes a key physiological pathway for K^+ secretion into the urinary fltrate. See text for additional details.

BK: Big or large conductance calcium-activated K^+ channel; ENaC: Epithelial Na^+ channel; NKCC2: Na^+-K^+-2Cl co-transporter; ROMK: Renal outer medullary K^+ channel.

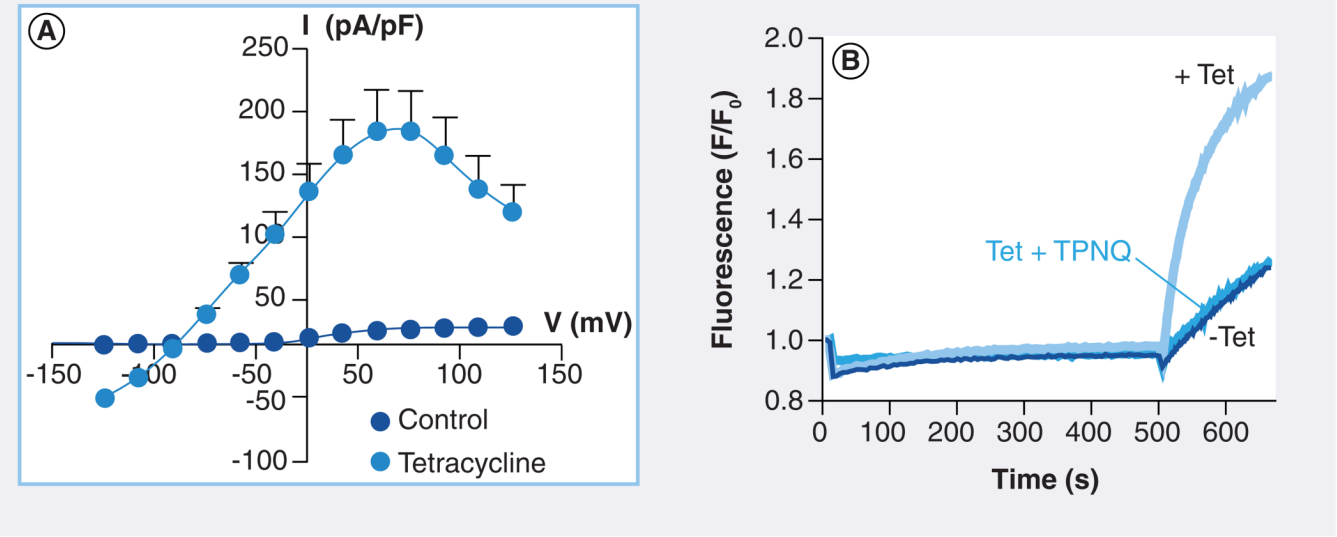

Figure 3. Thallium-fux assay of Kir1.1 channel function for high-throughput molecular library screening

(A) A stable cell line expressing Kir1.1 under the control of a tetracycline-inducible promoter was developed to avoid cytotoxic effects of constitutive Kir1.1 channel expression and subsequent cell line degeneration. Values are means ± SEM current amplitude normalized to cell capacitance and plotted as a function of test voltage in whole-cell patch clamp experiments. Cells were cultured overnight in the absence (control; darker circles) or presence of tetracycline (lighter circles). **(B)** Fluorescence assay of Kir1.1 channel activity in a 384-well plate using the thallium-sensitive fluorescent dye FluoZin-2. Cells were cultured overnight in the absence (− Tet) or presence (+ Tet) of tetracycline. One well was pretreated with the Kir1.1 channel inhibitor peptide Tertiapin-Q (+ TPNQ) to block channel activity. Addition of extracellular thallium (at 500 s) evoked an abrupt increase in FluoZin-2 fluorescence that was dependent on Kir1.1 channel expression. This assay was used to screen approximately 225,000 small molecules for novel modulators of Kir1.1.

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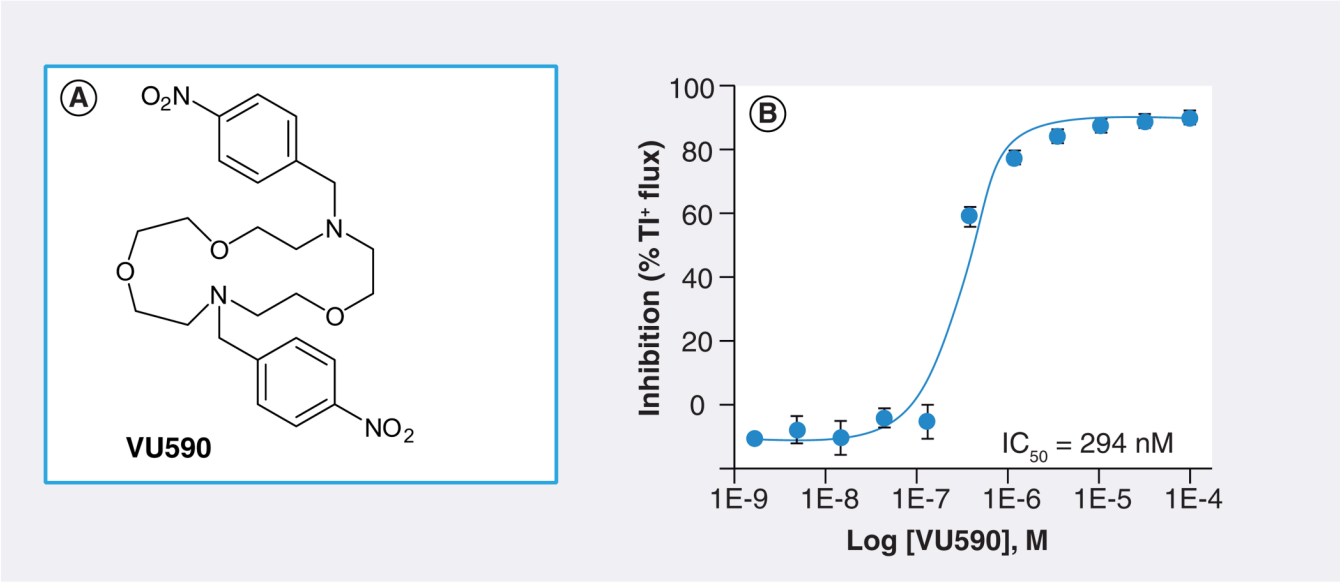

Figure 4. VU590, the first disclosed small-molecule inhibitor of Kir1.1

(A) Molecular structure of VU590. **(B)** Concentration–response curve for VU590 inhibition of Kir1.1 recorded in thallium flux assays.

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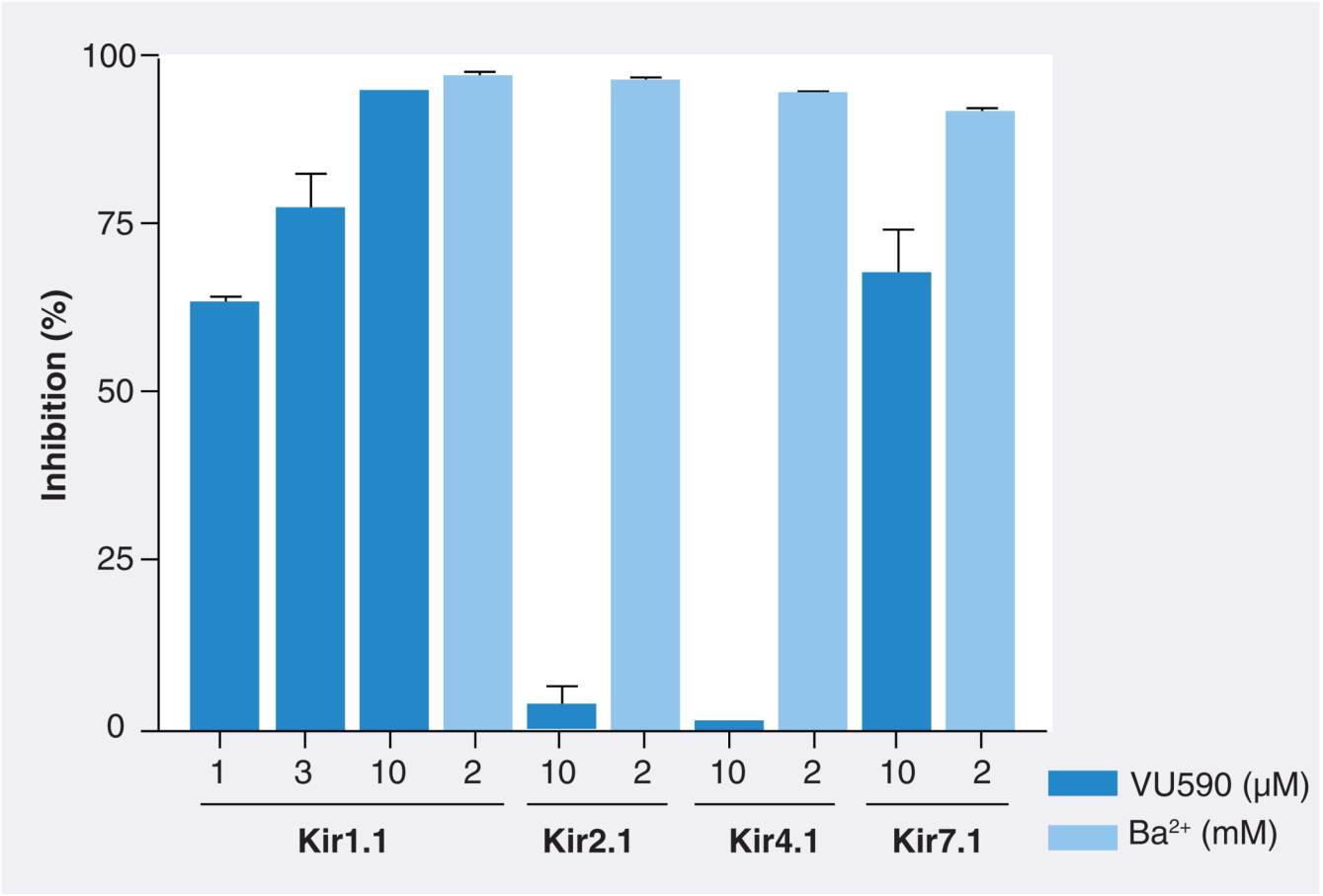

Figure 5. Selectivity of VU590 among members of the Kir channel family

Values are mean ± standard error percentage inhibition of the indicated Kir channel by VU590 (darker bars) or Ba^{2+} (lighter bars). The concentrations of VU590 (μM) and Ba^{2+} (mM) used are shown below each bar. Current amplitude was recorded at −120 mV.

Reproduced with permission from [47].

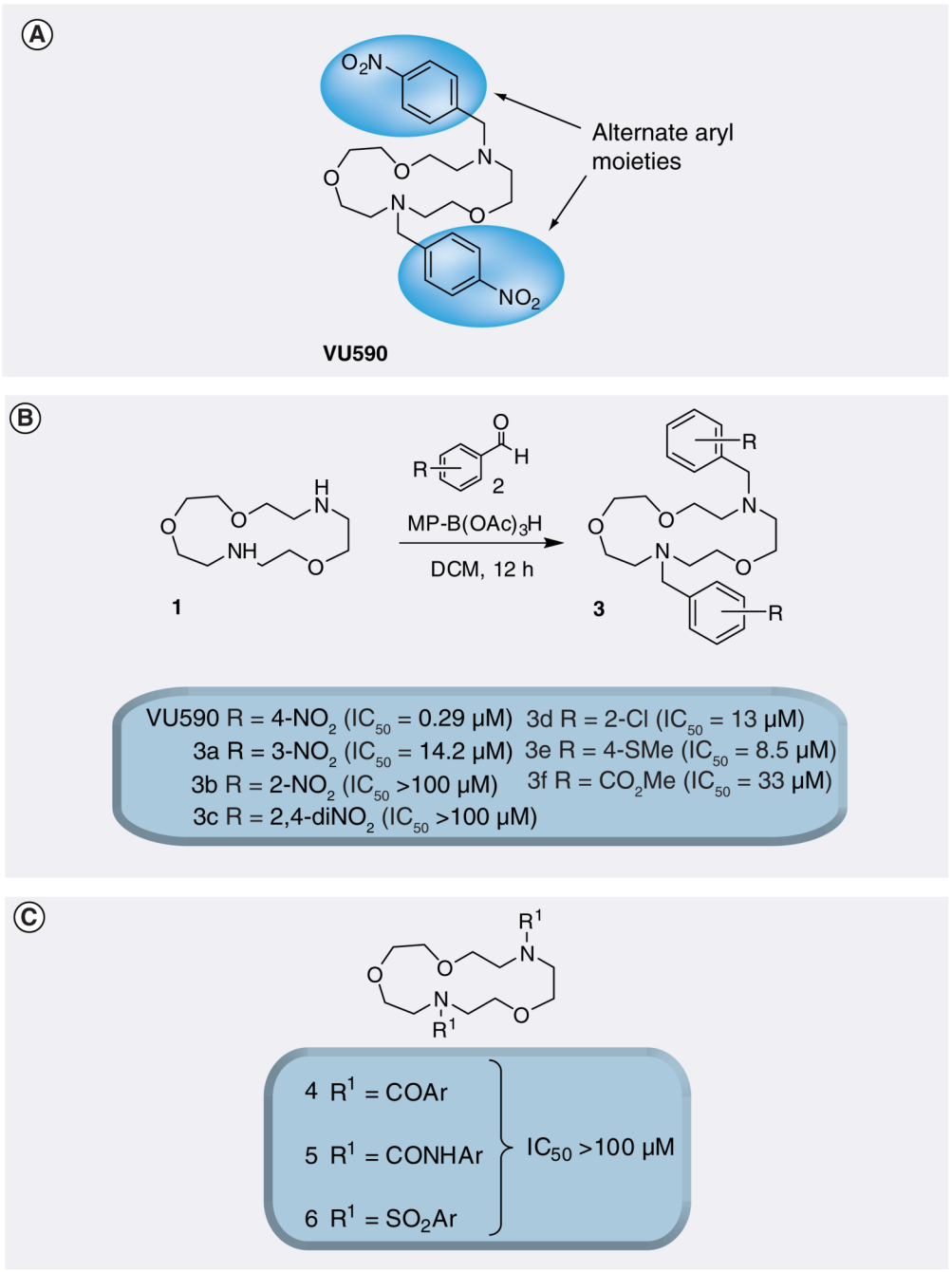

Figure 6. Determination of VU590 structure–activity relationships through combinatorial chemistry and thallium-flux assays

(A) VU590 and areas to explore through chemical optimization. **(B)** Structure–activity relationship of VU590 analogs. **(C)** Alternate capping agents used to attenuate the basicity of VU590.

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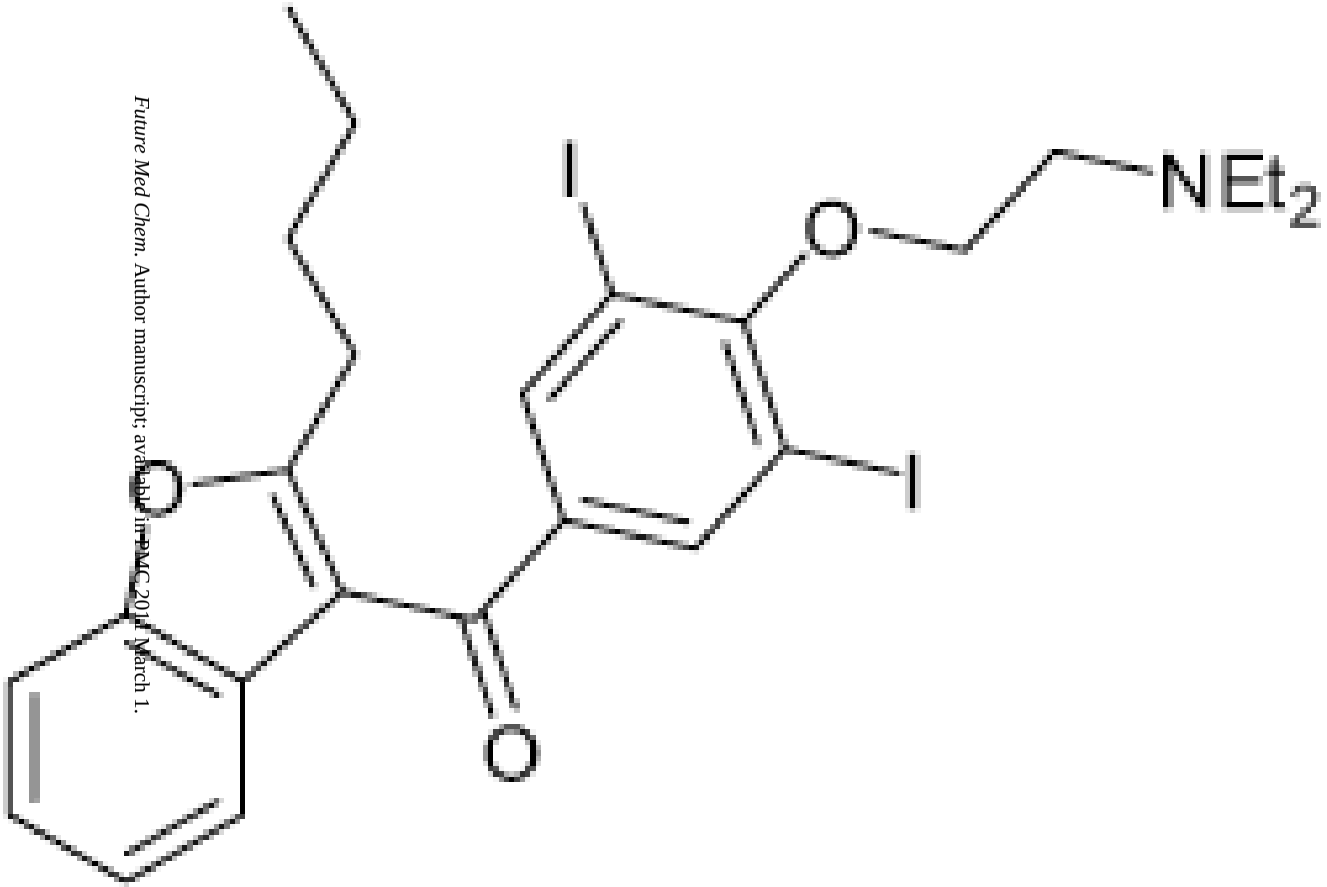
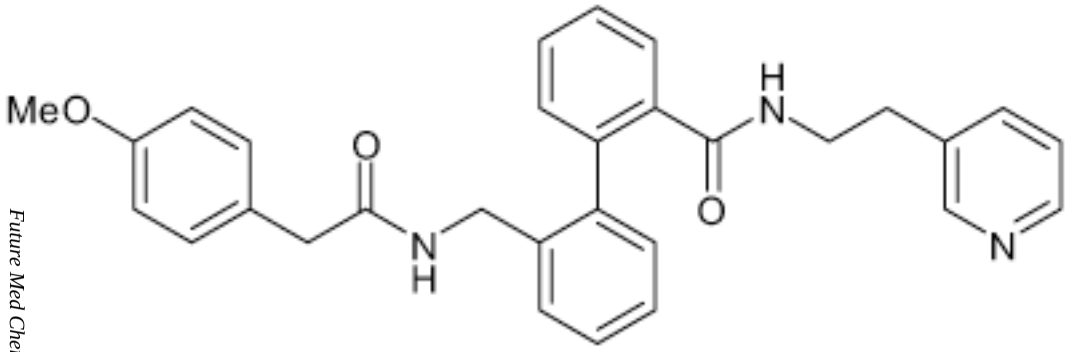
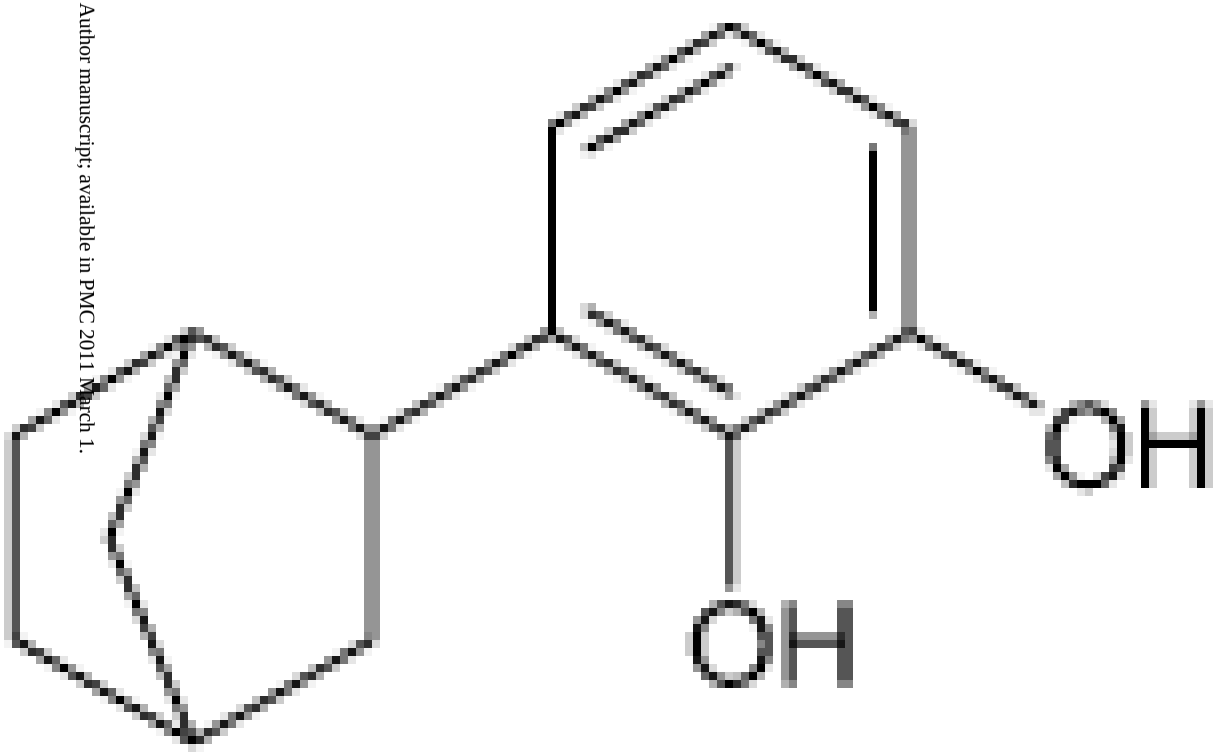
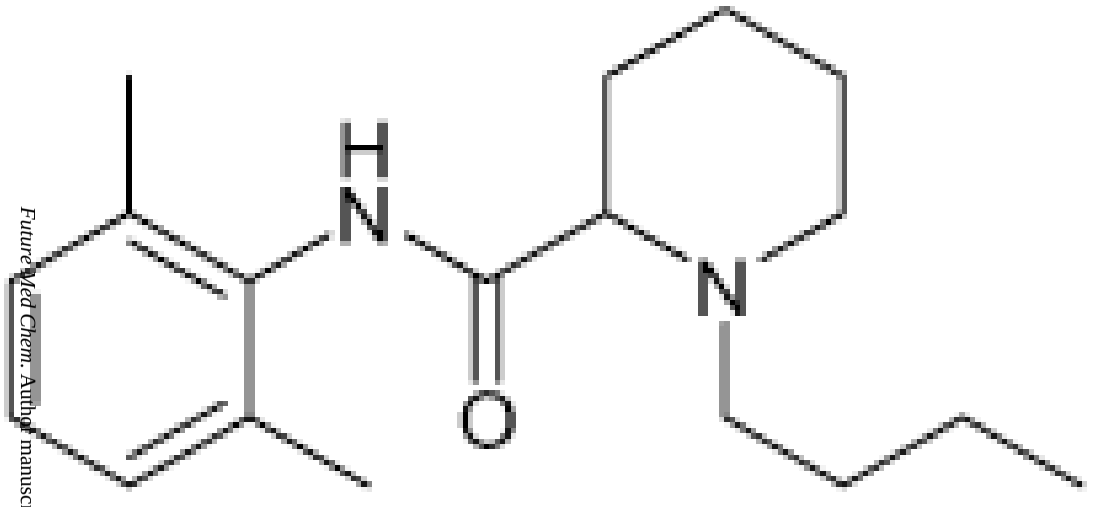
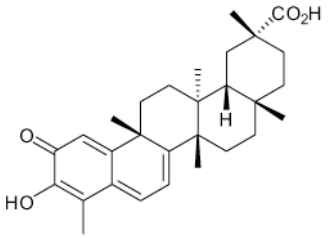
Table 1

ed small-molecule Kir channel modulators.

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	I_{KACh}	1.8–2.4 μM	Known voltage-gated Na, K and Ca channel blocker	[78]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	I_{KACh}	4.5 μM	Known voltage-gated K channel blocker	[79]
	Kir2.1	60 μM	Kv2.1 > Kir2.1	[58]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3	22–170 μM[†] Gβγ and Voltage dependent	>Kir 1.1, 2.1 Known voltage-gated Na and K channel blocker	[91]
	Kir2.1	>20 μ M channel activity 1.2 μ M channel traffcking	hERG > Kir2.1 > Kv2.1	[61]

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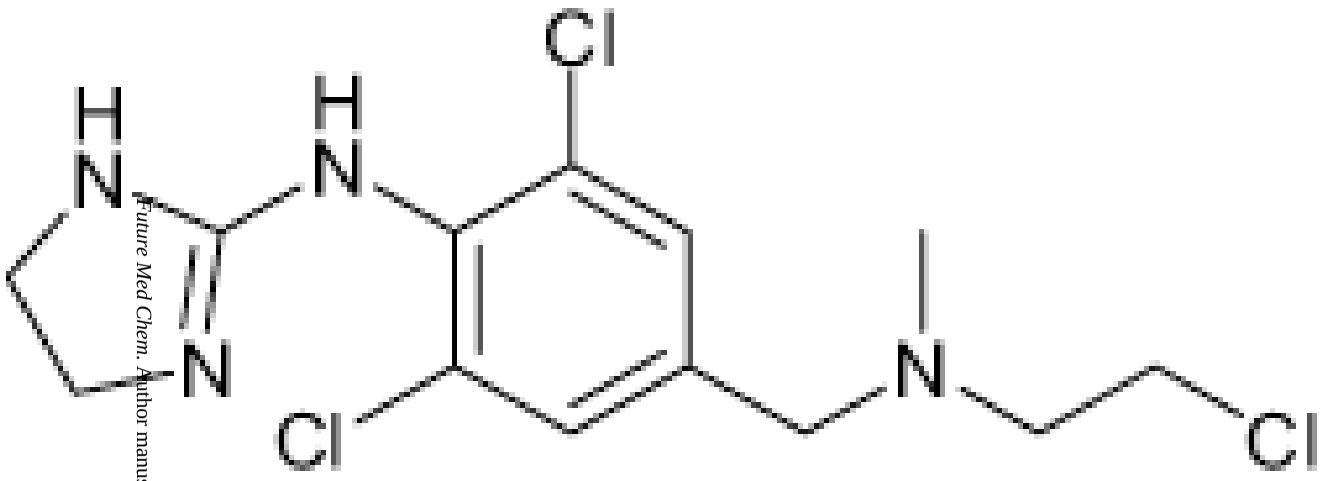
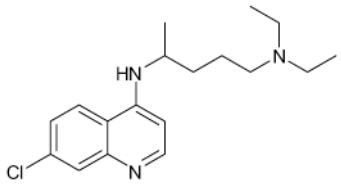
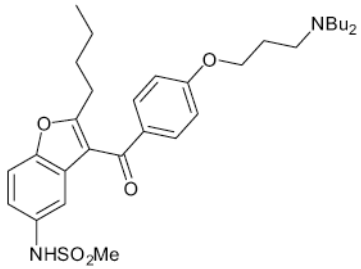
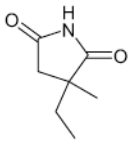
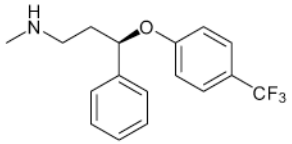
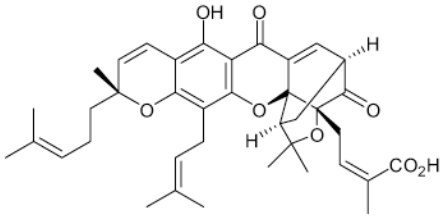
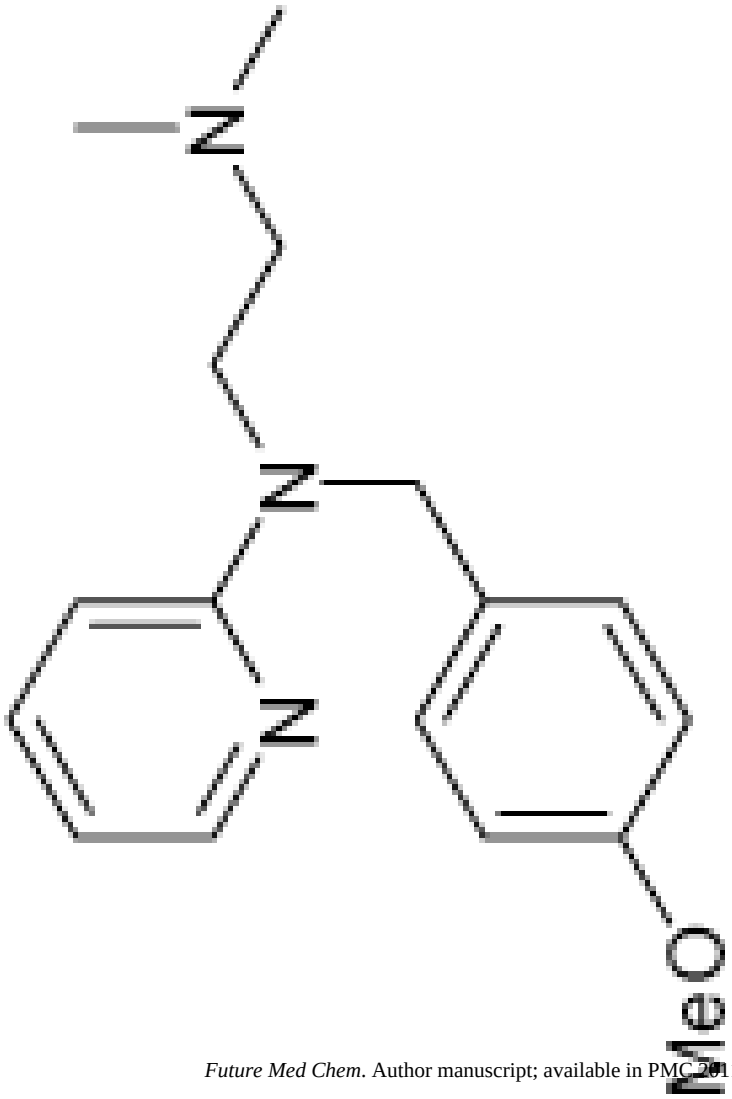
	Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
ne		Kir2.1	37 μM	Known α2 adrenergic agonist	[62]
		Kir2.1	8.7 μ M; Voltage dependent	Unknown	[13]

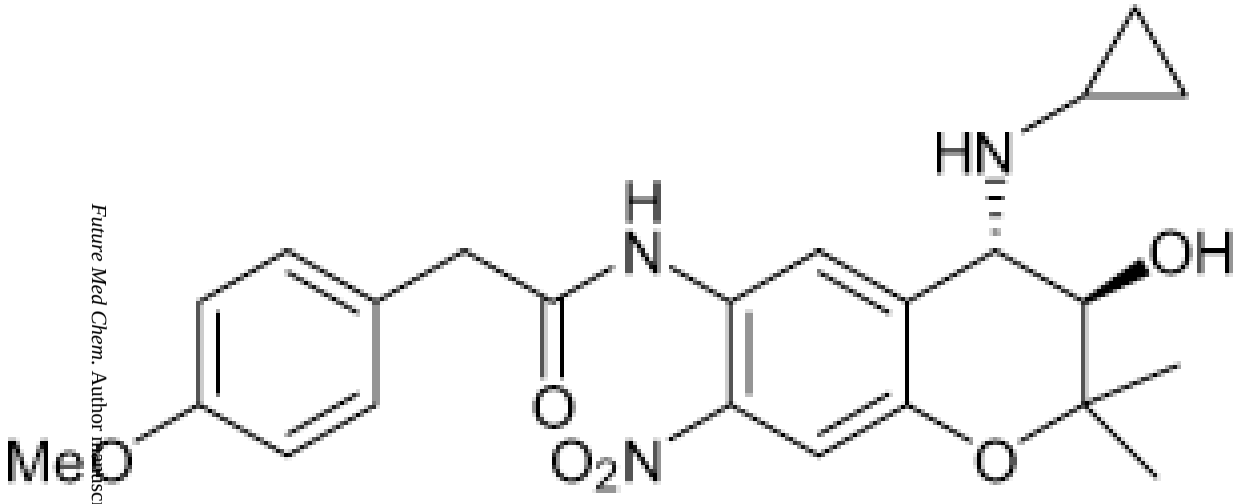
Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir2.3	689 μM[†] (~80%)	>Kir 2.1 Known antihistamine	[63]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	I_{KACh}	10–63 nM	Known voltage-gated Na, K and Ca channel blocker	[76]
	Kir3	0.4–3.5 mM† (61–76%)	>Kir 1.1, 2.1 Known voltage-gated Ca and Na channel blocker	[92]
	Kir3 Kir4.1	7–13 μM† (58–74%) 15.2 μM	>Kir 1.1, Kir 2.1 Known selective serotonin receptor inhibitor	[90,111]
	Kir2.1	>10-μM acute application (<50%) 27 nM chronic application 3h	Kv2.1>Kir2.1>Kir1.1 ~hERG	[59]

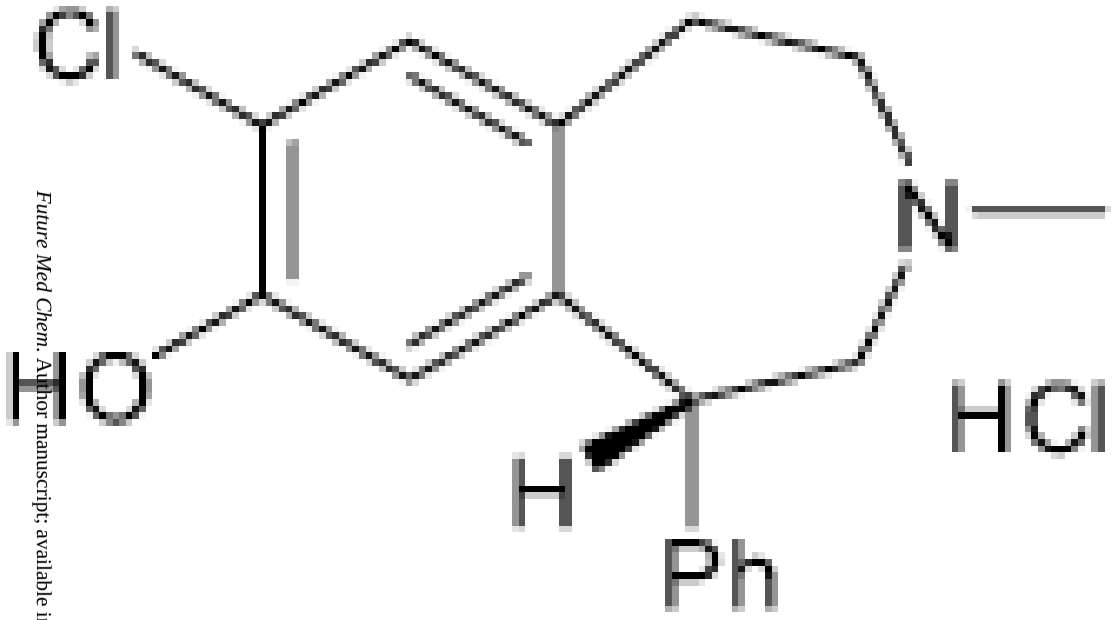
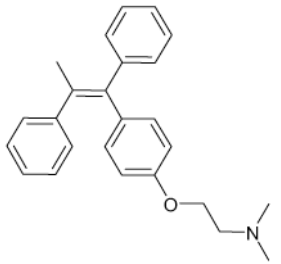
Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3	60 μM[†]	Unknown	[93]

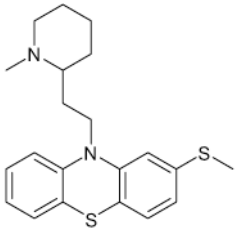
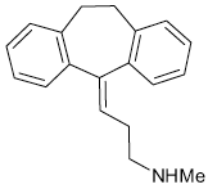
Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	I_{KACh}	0.1–2.5 μM	Known voltage-gated Ca, Na and K channel blocker	[77]

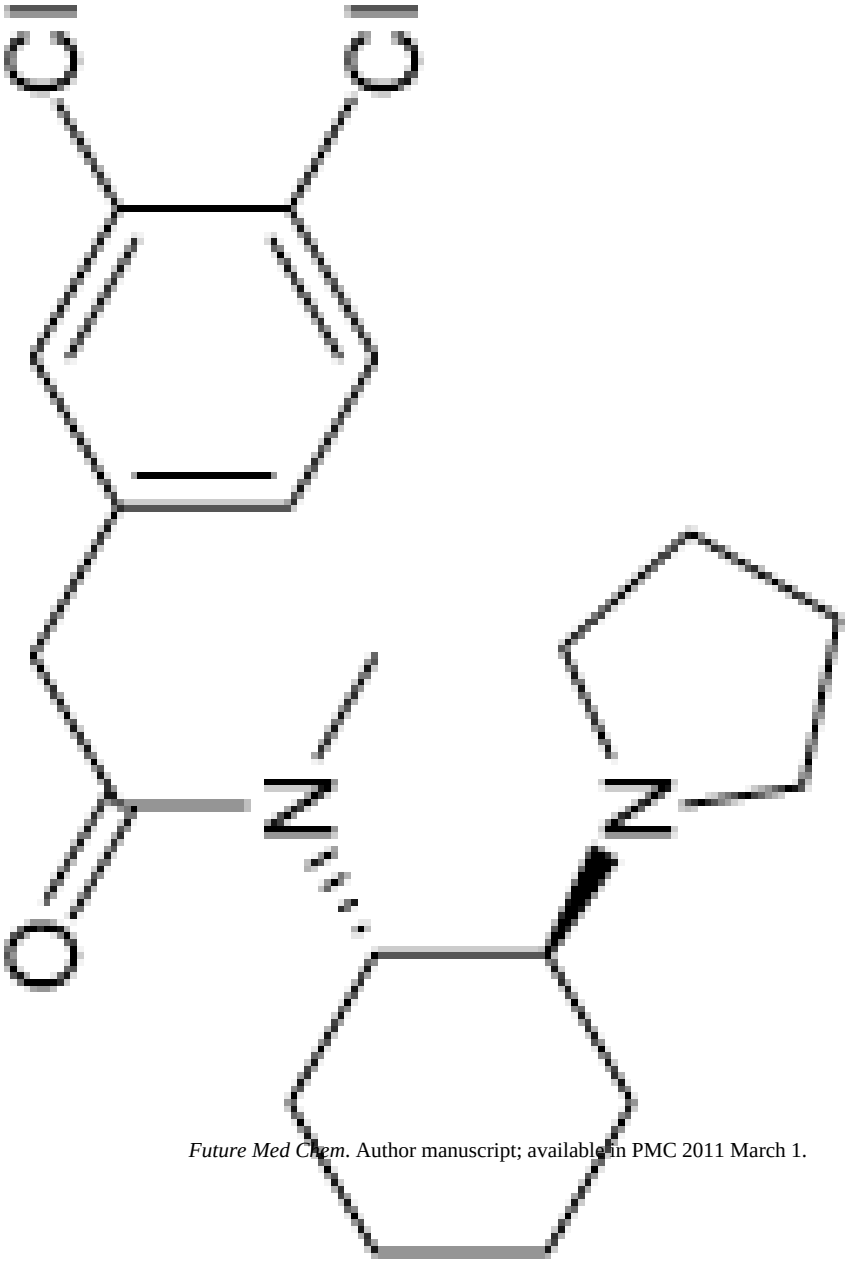
Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir2.3 Kir3.4	306 μM† (80%) <300 μM† (not reported)	>Kir 2.1 Known antihistamine	[63]

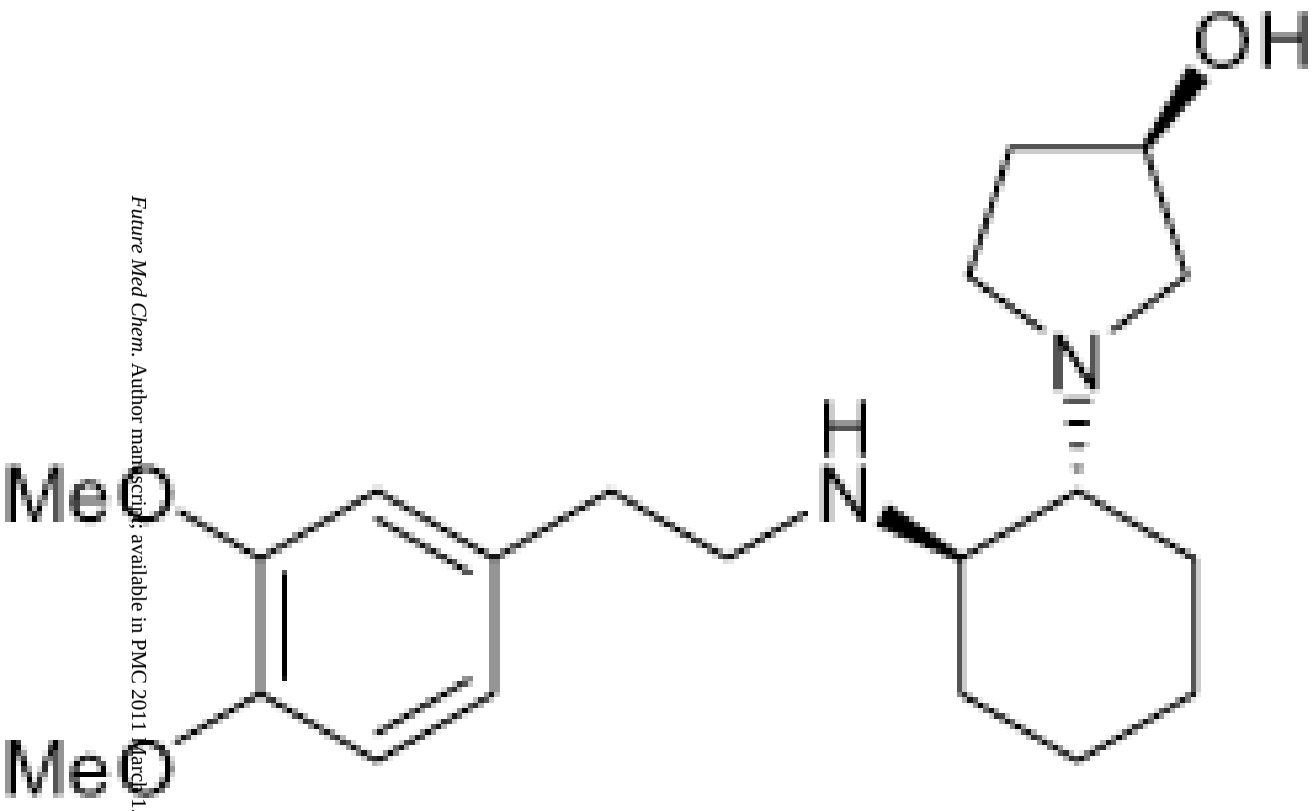
Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3.1/3.4	0.64 μM	Unknown Blocks Kv1.5 with similar potency	[80]
Structure not disclosed	Kir3.1/3.4	1.6 nM	Unknown HERG IC_{50} 58 μM	[81]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir2.3	EC_{50} 16 μM[†] (80%)	>Kir1.1, 2.1, 3.1/3.2	[65]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3	0.3–8 μM	Known D1 receptor antagonist	[94]
	Kir2	0.3–0.9 μM	Unknown Known estrogen receptor antagonist	[64]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3	58 μ M (92%)	Unknown Known antipsychotic	[89]
	Kir3 Kir4.1	18–71 μM† (53–76%) 16–38 μ M; voltage dependent (100%)	>Kir 1.1, 2.1	[88,110]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	Kir3	70 μM[f]	Known κ opioid receptor agonist	[95]

Structure	Kir Target	IC_{50} (max)	Selectivity	Ref.
	I KACh	10 μM	Known blocker of multiple voltage-gated Na and K channels	[76]

ntrations (IC_{50}) are listed with percent maximal inhibition in parentheses, if full block was not observed.

d for Kir channels expressed heterologously in Xenopus oocytes, in which significantly higher small-molecule IC_{50} values have been observed when compared with mammalian cells