DEATH BY QT: A NEW SAFETY CHALLENGE

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DECLARATION

The work reported in this thesis was carried solely by the candidate at RMIT University through the School of Medical Sciences whilst in the receipt of the Medical Advances Without Animals (MAWA) Trust Doctoral Scholarship.

This thesis contains no material which has been accepted for the award of any degree or diploma in any university. To the best of my knowledge and belief, this thesis contains neither material previously published or written by another person nor experimental data from another person’s work except where due reference is made in the text of the thesis. The content of this thesis is the result of work which has been carried out since the official commencement date of the approved research program. This thesis is less than 100,000 words in length, exclusive of tables, figures and references.

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SUMMARY

1. The HERG gene encodes for the delayed rectifier K⁺ channel in human cardiac tissue and highly contributes to the repolarization phase of the ventricular action potential. It underlies two cardiac disorders linked to a prolongation in the QT interval which are classified as either congenital or acquired long QT syndrome. The channel has been the cause of concern in the past decade due to its structural properties that lead to its unintentional inhibition by various classes of drugs. This unintentional inhibition has lead to the development of testing guidelines for pharmaceuticals in vivo and vitro to detect this effect prior to clinical testing. To date, no assay has been set as a standard due to variability across laboratories, the use of animals providing unreliable results and due to the unspecificity of the assays developed. This thesis focuses on the development of testing assays for HERG using animal-free methodology.

2. In Chapter 2, a human embryonic kidney (HEK293) cell line was transfected with the HERG gene using animal-free methodologies. The cell line was cultured using human serum in placement for foetal calf serum. The transfection reagent used was lipofectamine 2000 CD, an animal-free lipophilic reagent. The success of the transfection was confirmed using PCR, patch clamp electrophysiology and a non-radioactive rubidium assay which mimics K⁺ ion transport properties. Using specific HERG primers, the RNA in transfected cells was reverse-transcribed and amplified using PCR. Bands at an expected size of 575 bp in the HERG transfected HEK293 cells confirmed HERG presence in comparison to the native HEK293 cells. The electrophysiological properties of HERG K⁺ currents were examined in HERG transfected HEK293 cells using patch clamp electrophysiology. HERG activation was apparent at voltages positive to -40 mV and showed inward rectification at voltages positive to -10 mV. The V₁/₂ of the fitted activation curve of HERG tail currents was -23.0 mV. These currents were not seen in the native HEK293 cells. Using a non-radioactive rubidium assay, several known HERG inhibitors were tested. The IC₅₀ values obtained were compared to those obtained using
electrophysiological studies in the literature. The correlation between these data and previously published values resulted in a correlation coefficient \( r^2 \) of 0.76.

3. In Chapter 3, a human neuroblastoma cell line (SH-SY5Y) was tested for its validity for testing the effect of drugs on the HERG K\(^+\) channel. The cell line endogenously expresses the HERG protein as part of its physiological function. Using specific HERG primers, the RNA in the neuroblastoma cells was reverse-transcribed and amplified using PCR. Bands at an expected size of 575 bp in the neuroblastoma cells confirmed HERG presence showing the same band detected in HERG transfected HEK293 cells. A non-radioactive rubidium assay was used to confirm the function of the HERG channels in the cell line. To eliminate efflux caused by K\(^+\) channels other than HERG, a cocktail of K\(^+\) channel blocking drugs were tested. Tetraethylammonium (TEA) was the only inhibitor of K\(^+\)-induced Rb\(^+\) efflux in SH-SY5Y cells which had no HERG effects. Ten known HERG inhibitors were tested in the presence of TEA (10 mM). The IC\(_{50}\) values obtained using the Rb\(^+\) assay were compared to those obtained from patch clamp studies in HERG transfected HEK293 cells in the literature and were found to be well correlated with a correlation coefficient value of 0.82.

4. Clomipramine causes prolongation in the QT interval in patients with depression; however its mechanism of action on cardiac cells leading to this cardiotoxic effect has not been defined as yet. In this study, clomipramine was tested using three different approaches that were developed in previous chapters of this thesis. These included testing in HERG transfected HEK293 cells and the neuroblastoma cell line (SH-SY5Y) using a rubidium assay. The drug was also tested using whole cell patch clamp. The results of the study indicate that clomipramine is an inhibitor of HERG as it resulted in an IC\(_{50}\) value of 8.35 \( \mu \)M and 2.18 \( \mu \)M testing in HERG transfected HEK293 cells and the neuroblastoma cell line (SH-SY5Y) using a rubidium assay respectively. Clomipramine inhibited HERG currents with an IC\(_{50}\) value of 0.50 \( \mu \)M using the patch clamp technique. The results indicate that the prolongation in the QT interval caused by the drug may involve HERG inhibition.
5. Protein kinases regulate several proteins including ion channels. The HERG K⁺ channel is regulated by several protein kinases including protein kinase A and protein kinase B. Only limited studies to date have examined HERG K⁺ current regulation by protein kinase C (PKC). In Chapter 5, the specific PKC activator and phorbol ester PDA was used to study HERG regulation by PKC in HERG transfected HEK293 cells. PDA caused a reduction in HERG currents in HEK293 cells at 1 and 20 µM. The PKC pseudo substrate inhibitor PKC [19-36] did not inhibit the effect of PDA on HERG currents. It also had no effect on HERG currents when tested in the absence of PDA. The results of the study suggest that (1) PDA could be acting directly on the channel and inhibiting its function or (2) PDA is activating other proteins which are affecting HERG currents in the HERG transfected HEK293 cells.
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I would also like to thank ASCEPT for all the financial support, which helped me to attend international conferences. I would also like to acknowledge the Society for allowing me to present my work during poster sessions and symposiums.

Finally, I dedicate this thesis to everyone in my family mum, dad, Heba, Hadeel and Ahmed. Your belief in me and your patience during difficult times made this period of my life easier.
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PUBLICATIONS ARISING FROM STUDIES DESCRIBED IN THIS THESIS

Communications to Scientific Meetings


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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
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<td>CHO</td>
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<td>DNA</td>
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<td>Human epithelial kidney 293</td>
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<tr>
<td>Rb(^+_r)</td>
<td>Rubidium</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>V(_{1/2})</td>
<td>Half maximum voltage</td>
</tr>
</tbody>
</table>
Chapter 1 General introduction

1.1 Cell structure and function

Cells, the building blocks of tissues contain machinery to divide and grow. They also contain machinery to perform specific functions such as contraction in the case of a muscle cell, transport of electrical signals in the case of a nerve cell, transport of oxygen to organs from the lungs by red blood cells, protection of the body from infections by white blood cells and the prevention of chemicals and microorganisms from entering the body by skin epithelial cells (Vander, 1998). All mammalian cells consist of a bilayer of phospholipids surrounding a cytoplasm in which the nucleus and other organelles are located. Cells have a potential difference across the plasma membrane due to an uneven distribution of ions and some cells such as muscle and nerve cells are excitable in that changing the membrane potential can lead to cellular activation. The following section (1.1.1) describes the nature of the membrane potential and how it is regulated.

1.1.1 Membrane potential

Plasma membranes consisting of a phospholipid bilayer surrounding cells and act as barriers separating the interior from the extracellular fluid (Bar, 1966). This bilayer controls the entry of molecules and maintains the electrical potential difference across the cell by acting as a barrier for ions such as potassium (K⁺), sodium (Na⁺) and chloride (Cl⁻). These ions carry charge and their unequal distribution across the cell membrane causes charge separation leading to a potential difference across the cell membrane. This internal ionic environment is maintained by a variety of means.
including membrane proteins which act as ion pumps, ion exchangers and ion channels. In most cells the membrane potential difference is between -50 and -90 mV between external and internal compartments. This potential difference is the result of the production of a range of intracellular anionic species such as proteins, polypeptides and organophosphates including phosphates and creatinine which cannot pass through the plasma membrane. The negative charge caused by the presence of such molecules is counterbalanced by an outflow of K⁺ ions across the membrane at resting conditions, due to the opening of potassium leak channels (Lesage, 2003; O’Connell, 2002). Some Na⁺ ion channels are also open but their activity at rest is low in comparison to the K⁺ ion channels. Thus K⁺ ions are concentrated in the inside of cells compared to the outside. The intracellular K⁺ concentration reaches equilibrium when its chemical concentration force due to its concentration gradient equals electrical force that drives K⁺ into the cell; this is known as the K⁺ equilibrium potential, see review by (Wright, 2004). The equilibrium potential can be calculated using the Nernst equation. The Nernst equation was first derived in 1888 by a German physical chemist (Edsall, 1974). The equation is used to calculate each ion’s equilibrium potential:

\[
E_x = \frac{RT \ln [X]_o}{zF} \frac{zF}{[X]_i}
\]

Where \([X]_o = \) ion outside, \([X]_i = \) ion inside, \(R\) the gas constant, \(T\) temperature (in degrees Kelvin), \(z\) the valence of the ion, \(F\) the Faraday constant.

The cell also contains other processes for eliminating intracellular Na⁺ in which under normal conditions is maintained at a low level within the cells. Na⁺ is maintained at a low level by the activity of the Na⁺-K⁺ ATPase (Figure 1-1). The
Chapter 1: General Introduction

pump actively transports Na\(^+\) out of the cell into the extracellular fluid in exchange for K\(^+\) ions from the extracellular compartment, see review by (Lopina, 2000). The pump requires the hydrolysis of ATP as its energy source to actively transport ions against their concentration gradient. The opening of selective Na\(^+\) ion channels allows extracellular Na\(^+\) to enter the cell down its electrochemical gradient and thus reduces the membrane potential and depolarizes the cell. The opening of membrane Ca\(^{2+}\) channels after depolarization can rapidly increase intracellular Ca\(^{2+}\) and in some cases also lead to depolarization. Ca\(^{2+}\) ions are a key activator of intracellular components and it is kept at low levels by Ca\(^{2+}\) sequestration into intracellular organelles such as endoplasmic reticulum and mitochondria, by the extrusion of Ca\(^{2+}\) through Ca\(^{2+}\) ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger (Missiaen, 1991). Entry of Na\(^+\) ions into the cell (during AP upstroke) is normally terminated prior to the cell membrane potential reaching the Na\(^+\) reversal potential (typically \(~\pm 60\) mV) and this is due to the rapid onset of inactivation of the voltage-gated sodium channels in addition to opening of K\(^+\) channels. This depolarization leads to the opening of K\(^+\) channels leading to the efflux of K\(^+\) and the restoration of the membrane potential. These ion movements represent the action potential which can be triggered in an all-or-nothing manner (see section 1.1.3).
Chapter 1: General Introduction

Figure 1-1: Ions channels in plasma membranes

Simplified schematic diagram of the plasma proteins responsible for the passage of ions into and out of a cell. The Na⁺-K⁺ ATPase transports Na⁺ out of the cell in exchange for K⁺ by the hydrolysis of ATP. The Na⁺/Ca²⁺ exchanger allows Ca²⁺ out of the cell in exchange with Na⁺. Other proteins in the plasma membrane are ion channels, which activate during cell excitation such as K⁺ ion channels, Na⁺ ion channels and Ca²⁺ ion channels. (Cr represents creatinine in the diagram).
1.1.2 Ion channels

Ion channels are composed of multiple integral proteins embedded within the phospholipid bilayer of cells (Singer, 2004). These proteins form a pore that is selective for specific ions (Unwin, 1993; Unwin, 1995; Weill, 1974) (Figure 1-2). Each protein subunit is formed of transmembrane domains that have a specific function in the activity of the ion channel such as detection of voltage changes, binding to specific neurotransmitters and other modulators, initiation of conformational changes in response to stimuli, as well as acting as gates (Armstrong, 1974; Lewis, 1990). The activity of an ion channel is dependent on certain stimuli; for example, subunits of voltage-gated ion channels have transmembrane domains that act as voltage sensors which contain aligned amino acids that respond conformationally to changes in the membrane potential and thus lead to the opening of the channel (Bezanilla, 2000; Guy, 1986; Schoppa, 1992). Channels may also contain binding domains for neurotransmitters which when occupied lead to either an increase or a decrease in the function of the ion channel. The state of channel opening and closing is known as gating. Gates can be activated due to external factors such as neurotransmitters, nucleotides, change in voltage, G-proteins and membrane pressure (Jiang, 2002; Jiang, 2003). The activity of ion channels is dependent on their activity status including activation, inactivation and deactivation (Vandenberg, 2004). For example, some voltage-gated K⁺ channels are closed at the resting membrane potential; a reduction in the membrane potential may lead to the opening of these channels causing an outflow of K⁺ ions from the cell (Vandenberg, 2001; Zhou, 1998a). These ion channels can become inactive during positive potentials for a period of milliseconds and re-activate again allowing K⁺ out of cells and finally go
through the deactive state once the original membrane potential is reached. The channel subunits contain transmembrane domains that cause conformational changes under certain stimuli, these changes allow the channel to become in an active state which allows ions through or can cause the channel to become deactivated which blocks the entry of ions into or out of the cell, see review by (Yu, 2004). The inactive state can be explained as a state where the channel is open to the intracellular compartments but is forming a shut gate to the extracellular side (Vandenberg, 2001).
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Figure 1-2: Ion channel structure

The structure of an ion channel found in the phospholipid bilayer of cells. **Panel A**: simplified model of the ion channel found in most diagrams. **Panel B**: Ion channels are usually composed of multiple identical subunits. The subunits aggregate to form a pore specific for the passage of certain ions. Each subunit contains 4 or greater transmembrane domains as in **Panel C**. One transmembrane domain in each subunit is responsible for the specificity of the ion passing through. **Panel D**: a model of a single domain and its integrated form within the phospholipid bilayer (Vander, 1998).
Ion channels act as selective gaps allowing the passage of specific ions across cell membrane (Figure 1-2) determined by the ion’s size and charge. For example, K⁺ is a large ion that attracts water less strongly as it is dispersed in its own charge. The dehydrated state of K⁺ allows it to pass through the plasma membrane pores, which are large enough to allow it through but too small to allow Na⁺ (since K⁺ charge density < Na⁺ charge density ⇒ hydrated K⁺ size > hydrated Na⁺ size) (Vlachy, 2004). In general, ion selectivity is determined by a combination of ion size and the energetics of dehydration relative to the energetic stabilization of the dehydrated ion within the ion selectivity filter (Noskov, 2005). These channels open and close depending on environmental changes that require specific ions to flow through. Ion channels exist in different forms which can be selective for certain ions such as Na⁺, K⁺, Ca²⁺ and Cl⁻. Some of these ion channels are voltage-gated and are unique as they can only be found in their open states during certain voltages.

Voltage-gated ion channels play a critical role in the control of the excitable state of a cell. These ion channels activate at certain membrane potential values to allow the entry or the exit of ions from cells. Voltage-gated ion channels are generally formed from multiple subunits which sense voltage changes, select certain ions and express sites for channel regulation by molecules such as toxins and drugs, see review by (Yu, 2004). To understand how these voltage-gated ion channels regulate cell function, their types, structure and function is reviewed below.
1.1.2.1  The voltage-gated Na\(^+\) channel family

Voltage-gated Na\(^+\) channels are expressed in excitable cells and consist of an \(\alpha\)-subunit encoding the functional channel and multiple \(\beta\)-subunits acting as auxiliary proteins which alter channel function (Isom, 1992). The \(\alpha\)-subunit of the ion channel is formed of 4 subunits containing six transmembrane domains. Multiple isoforms of the voltage-gated Na\(^+\) channels (Na\(_v\)) are found expressed in the nervous system including Na\(_{v1.1}\), Na\(_{v1.2}\), Na\(_{v1.3}\), Na\(_{v1.6}\), Na\(_{v1.7}\), which are sensitive to the neurotoxin tetrodotoxin (TTX) and Na\(_{v1.8}\), Na\(_{v1.9}\) and Na\(_x\) which are resistant to TTX. Other excitable tissues such as the skeletal muscle express the Na\(_{v1.4}\) isoform that is sensitive to TTX; and the heart muscle expresses the Na\(_{v1.5}\) and Na\(_x\) isoform which are resistant to TTX (Ogata, 2002).

The Na\(_{v1.1}\), Na\(_{v1.2}\) and Na\(_{v1.5}\) channels have very similar characteristics. These channels function along with the regulatory subunits \(\beta_1\), \(\beta_2\), \(\beta_3\), and \(\beta_4\) (Catterall, 2000a; Chen, 2004; Schaller, 2003). The channels are activated by veratridine, batrachotoxin, aconitine, grayanotoxin, and \(\beta\)-scorpion toxin; and are inhibited by tetrodotoxin, saxitoxin and antiarrhythmic drugs such as lidocaine (Wang, 1996a). The channels differ in location; Na\(_{v1.1}\) is expressed in the central nervous system; Na\(_{v1.2}\) channels are expressed in central neurons as well as cardiac myocytes; and Na\(_{v1.5}\) are expressed in cardiac myocytes (Rogart, 1989a), skeletal muscle (Trimmer, 1989a) and some brain neurons. The channels differ in their activation and inactivation potentials. Na\(_{v1.1}\) activates at -33 mV and inactivates at -72 mV (Clare, 2000); the Na\(_{v1.2}\) channels activate at -24 mV and inactivates at -53 mV; and the
Nav1.5 activation and inactivation parameters depend on the type of anions used in the intracellular recording solution (Li, 2002; Mantegazza, 2001; Sheets, 1999).

The Na\textsubscript{v}1.3 channels are expressed in central neurons and cardiac myocytes where the $\beta_1$ and $\beta_3$ subunits modulate the inactivation of the channel (Meadows, 2002). The activation voltage of the channel is at -23 mV and it inactivates at -65 mV (Cummins, 2001). The channel plays a critical role in the initiation of action potentials and repetitive firing. It is inhibited by the same drugs and toxins listed above for the Na\textsubscript{v}1.1 and Na\textsubscript{v}1.2 channels.

The Na\textsubscript{v}1.4 channels have different characteristics to other channels described. The channel is the only voltage-gated Na\textsuperscript{+} channel found to be expressed with a single $\beta_1$ regulatory subunit (Ferrera, 2006) and is found highly expressed in skeletal muscle (Filatov, 2005). The channel is selectively inhibited by $\mu$-conotoxin GIIIA and $\mu$-conotoxin PIIIA; other inhibitors include tetrodotoxin, saxitoxin, antiepileptic and antiarrhythmic drugs. The channel has an activation voltage of -26 mV and inactivates at -56 mV (Bennett, 2004).

The Na\textsubscript{v}1.6 channel is distributed in neurons found in the brain including the cerebellum, hippocampus, brainstem, spinal cord and many more (Burbidge, 2002; Whitaker, 2000). The channel has two regulatory subunits, the $\beta_1$ and $\beta_2$. The channel activation and inactivation parameters are species dependent and it is inhibited by tetrodotoxin, saxitoxin, antiepileptic and antiarrhythmic drugs.
The \( \text{Na}_v \)1.7 channel is expressed with two regulatory subunits, \( \beta_1 \) and \( \beta_2 \). The channel is inhibited by tetrodotoxin, saxitoxin, antiepileptic and antiarrhythmic drugs. It is distributed in dorsal root ganglia (DRG) neurons, sympathetic neurons and neuroendocrine cells (Klugbauer, 1995; Sangameswaran, 1997b). In DRG neurons the channel has an activation voltage of -45 mV and inactivation of -65 mV (Rush, 1998). The \( \text{Na}_v \)1.8 and \( \text{Na}_v \)1.9 channels are the only two channels that have not yet been associated with regulatory subunits. They are both tetrodotoxin resistant and are expressed in differing parts of DRG neurons (Renganathan, 2001). In rat DRG neurons, the \( \text{Na}_v \)1.8 channel has an activation voltage of -16 mV and inactivation voltage of -30 mV, whilst the \( \text{Na}_v \)1.9 channel has an activation voltage of -60 mV and -44 mV.

1.1.2.2 The voltage-gated \( \text{Ca}^{2+} \) channel family

Voltage-gated \( \text{Ca}^{2+} \) channels play a critical role in the facilitation of \( \text{Ca}^{2+} \) entry into the cytosol of cells. The channels play a critical role in regulating depolarization in excitable cells and cellular events including contraction, secretion, cell division and gene expression, see reviews by (Catterall, 2005; Yu, 2004). These channels consist of an \( \alpha_1 \)-subunit which forms the pore required for \( \text{Ca}^{2+} \) transport, and may have a range of multiple accessory subunits, depending on the type of tissue it is expressed in, playing a regulatory role in channel function, see review by (Dolphin, 1996). The \( \alpha_1 \)-subunit is composed of four subunits containing six transmembrane domains each and the channels termini are located in the intracellular compartment of cells. Five types of voltage-gated \( \text{Ca}^{2+} \) channels have been identified as L-type, N-type, P/Q type, R-type and T-type.
Excitable cells such as smooth muscle cells express the L-type Ca\(^{2+}\) channels required for excitation-contraction coupling (Flucher, 1996; Shirokov, 1998; Welling, 1997) along with the multiple accessory subunits, \(\alpha_2\delta\), \(\beta\) and \(\gamma\) (Cooper, 1987; Reimer, 2000; Shirokov, 1998; Takahashi, 1987). The L-type channels activate during strong depolarization and their activation is long lasting. They are inhibited by dihydropyridines, phenylalkylamines and benzothiazapines such as nifedipine, verapmil and diltiazem respectively (Godfraind, 1982; Striessnig, 1999; Welling, 1997). The channels are also found in neurons (Hell, 1993), cardiac pacemakers (Mangoni, 2003), atrial myocytes (Zhang, 2005), purkinje cells (Zhou, 1998b) and endocrine cells (Garcia-Palomero, 2001) where they activate at low voltages and play a critical role in neuronal synaptic regulation, cardiac pacemaking and release of hormones respectively.

The N-type Ca\(^{2+}\) channels are also found expressed with multiple subunits such as \(\alpha_2\delta/\beta_1\), \(\beta_3\), \(\beta_4\) and \(\gamma\). These channels regulate neurotransmitter release and hormone release in presynaptic terminals, dendrites and cell bodies of neurons (Westenbroek, 1992) and are activated by strong depolarization. The channel are blocked by \(\omega\)-conotoxin GVIA, \(\omega\)-conotoxin MVIIA and \(\omega\)-conotoxin GVIIC (Colledge, 1992).

The P/Q Ca\(^{2+}\) channels have similar characteristics and function as the N-type except that its regulatory subunits lack the \(\beta_3\) and \(\beta_4\). They are inhibited by \(\omega\)-conotoxin GVIIC (Mintz, 1992b).
The R-type channels are distributed in neuronal cell bodies, dendrites, heart, testes and the pituitary where they play a role in Ca\(^{2+}\) transients and repetitive firing (Dietrich, 2003a). The channels are blocked by SNX-482, mibefradil (Jimenez, 2000b) and volatile anesthetics (Kamatchi, 1999b). The channels regulatory subunits include \(\alpha_2\delta/\beta\), and \(\gamma\).

The T-type (transient) channels are activated by weak depolarization and are distributed in most parts of the brain (Perez-Reyes, 1998b) and are found in the heart (Cribbs, 1998; Zhou, 1998b), ovary and placenta depending on the isoform of the channel expressed. Some isoforms of the channel have expressed associated subunits (Perez-Reyes, 1998a) and some do not. These channels are inhibited by mibefradil (Martin, 2000), pimozide (Santi, 2002) and amiloride (Lacinova, 2000).

1.1.2.3 The voltage-gated K\(^+\) channel family

K\(^+\) channels are found in almost all tissues and play a critical role in maintaining the resting membrane potential of cells by allowing outflow of K\(^+\) thereby repolarizing a depolarized cell, maintaining the equilibrium potential of K\(^+\) near the resting membrane potential value, and controlling cell volume (Hille, 1992). K\(^+\) ion channels consist of four \(\alpha\) subunits lining the pore (Jan, 1992) and are regulated by an auxiliary \(\beta\) subunit (Jan, 1997). These channels are classified into 6 subfamilies depending on their function including Shab, Shaker, ether-a-go-go (EAG) K\(^+\) channels, Shaw, Shal and KCNQ K\(^+\) channels (Jan, 1997).
The ether-a-go-go (EAG) K⁺ channel family comprises channels that consist of four subunits each consisting of six putative transmembrane domains (S1-S6) with S4 domain acting as the voltage sensor (Bauer, 2001). The EAG family is subdivided into three classes, the eag, elk and erg channels, which are each subdivided further into different isoforms (Figure 1-3). The biological function of the channels varies according to the subclass of the channel. For example the eag K⁺ channels are found expressed in bovine photoreceptors and retinal ganglion cells (Frings, 1998), human myoblast cells (Bijlenga, 1998; Occhiodoro, 1998), human neuroblastoma (Meyer, 1998) and melanoma cells (Meyer, 1999). The erg channels are found expressed in various cell types including cardiac cells (Pond, 2000), the hippocampus and astrocytes (Emmi, 2000), smooth muscle (Akbarali, 1999), neuroblastoma cells (Arcangeli, 1999) and in lactotrophs (Bauer, 1998; Schafer, 1999). The elk channels are found expressed in testis, brain, lung, colon and pituitary gland (Engeland, 1998; Saganich, 1999) (Table 1-1).

The KCNQ channels have a similar structure to the other K⁺ channels containing four subunits of six transmembrane domains each (Robbins, 2001). To date, five subtypes of the channel have been identified as KCNQ1, KCNQ2, KCNQ3, KCNQ4 and KCNQ5. All channels in this family have been associated with members of the KCNE gene family to form a functional channel (Table 1-1). These include the KCNQ1 assembling with KCNE1 and KCNE3 (Melman, 2002; Wang, 1996b; Yang, 1997), the KCNQ2 assembling with KCNE1 and KCNE2 (Singh, 1998), the KCNQ3 assembling with KCNQ2, KCNQ4, KCNQ5 and KCNE2 (Charlier, 1998), the KCNQ4 assembling with KCNQ3 (Kubisch, 1999), and the KCNQ5 assembling with KCNQ3 (Lerche,
The individual channels are differentially expressed in different tissues. The channels are found expressed in various tissues such as heart, kidney, lung, testis, spleen, skeletal muscle, central nervous system, the pituitary and placenta (Table 1-1). Drugs that have an inhibitory effect on all KCNQ isoforms include tetraethylammonium, linopirdine and XE991 (Hadley, 2000; Robbins, 2001; Wang, 2000; Wang, 1998).

The Shaker-related K⁺ channel family includes two types of K⁺ channels, the fast-inactivation A-type currents and slowly inactivating delayed-rectifier type channels (Edwards, 1995). The structure is formed from six transmembrane domains forming the functional channel. These K⁺ channels are inhibited by tetraethylammonium and 4-aminopyridine (Mathie, 1998) (Table 1-1). The channels are mainly expressed in neurons and various tissues including brain tissue, heart tissue, testis, platelets, skeletal muscle, pancreatic islet, arteries, astrocytes, adrenal gland and lymphocytes, see review by (Gutman, 2005).

The Shab-related channel family has one type of voltage-activated K⁺ channels known as delayed rectifier channels, see review by (Gutman, 2005). These channels are found expressed in brain, cardiac cells, skeletal muscle, eye, lung, pulmonary artery, gastrointestinal smooth muscle and mesenteric artery smooth muscle (Brahmajothi, 1997; Frech, 1989; Hwang, 1992; Lee, 2003; Patel, 1997; Schmalz, 1998; Xu, 1999) (Table 1-1).
The Shal-related channel family is comprised of three channels, Kv4.1, Kv4.2 and Kv4.3. The channels structures are similar to those described above. The channels are highly expressed in the heart, brain and smooth muscle where they encode the A-type K$^+$ current, also known as transient currents (Brahmajothi, 1996; Dixon, 1996; Serodio, 1996; Tkatch, 2000) (Table 1-1). The channels are inhibited by 4-aminopyridine in the millimolar range (Pak, 1991; Tseng, 1996).

The Shaw-related family consists of delayed rectifier, A-type and fast inactivating A-type K$^+$ channels (Goldman-Wohl, 1994; Madeja, 2000; McCormack, 1990; Schroter, 1991). They are expressed in brain, skeletal muscle, lung and testis (Lien, 2002; Madeja, 2000) (Table 1-1). Several drugs inhibit the current encoded by the Shaw-related K$^+$ channels including diltiazem, tetraethylammonium, 4-aminopyridine, capsaicin, nifedipine, verapamil (Table 1-1).
Figure 1-3: The ether-a-go-go gene (EAG) K⁺ channel family.

The EAG family is subdivided into three subfamilies: eag, elk and erg. See section 1.1.2.3.
### Table 1-1: Summary of voltage-gated ion channel types.

<table>
<thead>
<tr>
<th>Channel family</th>
<th>Type</th>
<th>Regulatory subunit</th>
<th>Tissue distribution</th>
<th>Inhibitors</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Na_v$</td>
<td>Na\textsubscript{1.1}</td>
<td>$\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$</td>
<td>CNS, cardiac myocytes</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Clare, 2000; Maier, 2002; Westenbroek, 1989)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.2}</td>
<td>$\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$</td>
<td>Central neurons</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Kaplan, 2001; Noda, 1986; Westenbroek, 1989)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.3}</td>
<td>$\beta_1$, $\beta_3$</td>
<td>Central neurons, cardiac myocytes</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Chen, 2000; Maier, 2002; Meadows, 2002)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.4}</td>
<td>$\beta_1$</td>
<td>Skeletal muscle</td>
<td>$\mu$-conotoxin GIIIA, $\mu$-conotoxin PIIDA, tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Chahine, 1994; Trimmer, 1990; Trimmer, 1989a)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.5}</td>
<td>$\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$</td>
<td>Cardiac myocytes, skeletal muscle, brain neurons</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Hartmann, 1999; Rogart, 1989b; Satin, 1992; Trimmer, 1989b)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.6}</td>
<td>$\beta_1$, $\beta_2$</td>
<td>Neurons of cerebellum, hippocampus, spinal cord</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Dietrich, 1998; Tzoumaka, 2000)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.7}</td>
<td>$\beta_1$, $\beta_2$</td>
<td>Dorsal root ganglia neurons, sympathetic neurons, neuroendocrine cells</td>
<td>Tetrodotoxin, saxitoxin, anti-epileptic, antiarrhythmics</td>
<td>(Felts, 1997; Sangameswaran, 1997a)</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{1.8} Na\textsubscript{1.9}</td>
<td>Dorsal root ganglia</td>
<td>Tetrodotoxin resistant</td>
<td>Tetrodotoxin resistant</td>
<td>(Akopian, 1996)</td>
</tr>
<tr>
<td>$Ca_v$</td>
<td>L-type</td>
<td>$\alpha_2$, $\delta$, $\beta$, $\gamma$</td>
<td>Neurons, cardiac pacemakers, atrial myocytes, purkinje cells, endocrine cells</td>
<td>Dihydropyridine, phenylalkylamines, benzothiazapine</td>
<td>(Catterall, 2000b; Glossmann, 1990; Takahashi, 1987; Takimoto, 1997)</td>
</tr>
<tr>
<td></td>
<td>N-type</td>
<td>$\alpha_2 \delta$, $\beta_1$, $\beta_3$, $\beta_4$ and $\gamma$</td>
<td>Presynaptic terminals, dendrites, cell bodies of neurons</td>
<td>$\omega$-conotoxin MVIIA, $\omega$-conotoxin GVIIA, $\omega$-conotoxin GVIIIC</td>
<td>(Hillyard, 1992; Scott, 1996; Westenbroek, 1992)</td>
</tr>
<tr>
<td></td>
<td>P/Q</td>
<td>$\alpha_2 \delta$, $\beta_1$, $\gamma$</td>
<td>Neuronal cell bodies, dendrites, heart</td>
<td>$\omega$-conotoxin GVIIC</td>
<td>(Mintz, 1992a)</td>
</tr>
<tr>
<td></td>
<td>R-type</td>
<td>$\alpha_2 \delta$, $\beta$, $\gamma$</td>
<td>Neuronal cell bodies, dendrites, heart</td>
<td>SNX-482, mibebradil, volatile anaesthetics</td>
<td>(Dietrich, 2003b; Jimenez, 2000a);</td>
</tr>
<tr>
<td>Family</td>
<td>KCNQ:</td>
<td>T-type</td>
<td>4-aminopyridine and specific isoform inhibitors</td>
<td></td>
<td></td>
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<tr>
<td>-----------------------------</td>
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<td>-----------------------------</td>
<td>------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shaker-related family:</td>
<td>Various: KCNE1, KCNE3, KCNQ3, KCNE2, KCNQ2, KCNQ5</td>
<td>Heart, kidney, lung, testis, spleen, skeletal muscle, central nervous system, pituitary, placenta</td>
<td>Tetraethylammonium, linopirdine, XE991. KCNQ4 is inhibited by tetraethylammonium only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shal-related family</td>
<td>Various including: K,5.1, K,6.1-6.3, K,8.1, K,9.1-9.3</td>
<td>Brain tissue, heart tissue, testis, platelets, skeletal muscle, pancreatic islet, arteries, astrocytes, adrenal gland, lymphocytes</td>
<td>Tetraethylammonium, 4-aminopyridine and specific isoform inhibitors</td>
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<td></td>
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<tr>
<td>Shaw-related family</td>
<td>KChIP1</td>
<td>Brain, skeletal muscle, lung, testis</td>
<td>Tetraethylammonium, barium, 4-aminopyridine</td>
<td></td>
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<tr>
<td>Various</td>
<td></td>
<td>Brain, colon, heart, testis, smooth muscle, lung, liver, kidney</td>
<td>Diltiazem, tetraethylammonium, 4-aminopyridine, capsaicin, nifedipine, verapamil</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-aminopyridine, tetraethylammonium</td>
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</tbody>
</table>

**Kv**

- **T-type**
  - Heart, ovary, placenta
  - Various depending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**EAG**

- Retinal ganglion cells, human myoblast cells, human neuroblastoma, melanoma cells, cardiac cells, hippocampal, astrocytes, smooth muscle, lactotrophs

**KCNQ:**

- Heart, ovary, placenta
- Brain tissue, heart tissue, testis, platelets, skeletal muscle, pancreatic islet, arteries, astrocytes, adrenal gland, lymphocytes
- Various depending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**Shaker-related family:**

- Heart, ovary, placenta
- Various: KCNE1, KCNE3, KCNQ3, KCNE2, KCNQ2, KCNQ5
- Brain tissue, heart tissue, testis, platelets, skeletal muscle, pancreatic islet, arteries, astrocytes, adrenal gland, lymphocytes
- Various depending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**Shal-related family:**

- Brain, ovary, placenta
- Various: KCNE1, KCNE3, KCNQ3, KCNE2, KCNQ2, KCNQ5
- Retinal ganglion cells, human myoblast cells, human neuroblastoma, melanoma cells, cardiac cells, hippocampal, astrocytes, smooth muscle, lactotrophs
- Various depending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**Shaw-related family:**

- Brain, ovary, placenta
- Various: KCNE1, KCNE3, KCNQ3, KCNE2, KCNQ2, KCNQ5
- Retinal ganglion cells, human myoblast cells, human neuroblastoma, melanoma cells, cardiac cells, hippocampal, astrocytes, smooth muscle, lactotrophs
- Various depending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**KChIP1**

- Brain, colon, heart, testis, smooth muscle, lung, liver, kidney
- Variousdepending on isoform: Quinidine, astemizole, terfenadine, dofetilide, barium

**References:**

- (Kamatchi, 1999a)
- (Bohn, 2000)
- (Lerche, 2000; Robbins, 2001)
1.1.3 **Generation of action potentials in excitable cells**

Excitation of excitable cells is caused by the entry of positively charged ions into the cytoplasm which causes a change in the membrane potential (Hille, 1992). These changes in the membrane potential occur over a period of milliseconds and are collectively known as action potentials (Hodgkin, 1952d). Action potentials consist of several phases describing different membrane events that occur over time including depolarization, repolarization and hyperpolarization. Excitable cells differ in the shape of the action potential depending on which ion channels control the movement of ions. For example, neurons transmit signals quickly and most of the ion channels that are found expressed are Na\(^+\) and K\(^+\) channels (Hodgkin, 1952a) (**Figure 1-4**). The action potential of a neuron consists of three phases including depolarization caused by the opening of Na\(^+\) channels allowing Na\(^+\) ions into the cell (Hodgkin, 1952b), once the membrane potential is reduced, K\(^+\) channels are opened allowing K\(^+\) ions to flow out leading to repolarization (Hodgkin, 1952c); and finally the hyperpolarization phase is due to the over flow of K\(^+\) ions causing the membrane potential to reach a value below its origin and therefore is brought back to its resting value (**Figure 1-4**). During the peak of Na\(^+\) efflux, Ca\(^{2+}\) ions enter into cells through voltage-depenent Ca\(^{2+}\) channels (Baker, 1971; Frankenhaeuser, 1957). Ca\(^{2+}\) is the trigger for a range of cellular events including enzyme activation and muscle contraction. Unlike neurons, the action potential of cardiac cells is triggered by the activation of several ion channels responsible for several phases of the action potential (**Figure 1-4**) which will be described in the following section.
Chapter 1: General Introduction

Figure 1-4: Action potentials of excitable cells.

Panel A: A schematic diagram of a neuronal action potential. The action potential consists of two major phases caused by the opening of Na\(^+\) and K\(^+\) ion channels.

Panel B: A schematic diagram of a cardiac action potential. The action potential consists of several phases caused by the opening of multiple ion channels including voltage-gated Na\(^+\) channels, Ca\(^{2+}\) channels and K\(^+\) channels. Phase 0 of the action potential represents Na\(^+\) entry into cells causing depolarization. Phase 1 is caused by Na\(^+\) channel inactivation and Ca\(^{2+}\) entry into the cells. Phase 2 is caused by the entry of Ca\(^{2+}\) opposed by K\(^+\) efflux. Phase 3 represents repolarization caused by the opening of the delayed rectifier K\(^+\) channels which allow K\(^+\) efflux. Phase 4 is the resting membrane potential and is caused by the inward rectifier channels (Vander, 1998).
1.1.4 Cardiac ion channels and their role in cardiac function

The heart is composed of a muscular sac located in the chest and functions to pump blood to tissues providing nutrients to maintain normal body functions. The human heart is divided into two sections each containing an atrium and a ventricle, separated by atrioventricular valves keeping the blood flow in one direction (Burch, 1985). The heart is composed of cardiac muscle composed of excitatory muscle cells whose contraction is initiated by depolarization. The initiation of cardiac contraction is caused by electrical activity initiated by depolarization at sinoatrial node cells which are located in the upper part of the left atrium, spreading through the atria and into the ventricles (Gaskell, 1883). This occurs where the rapid rate of spontaneous depolarization of the sinoatrial node cells triggers action potentials in the atrial muscle cells and in bands of connected cells known as the atrioventricular node, see review by (Kaupp, 2001). This depolarization travels through the conduction system that includes the Bundle of His, right and left bundle branches and the Purkinje fibres (Figure 1-5). The electrical signals in the Purkinje fibres are then spread into the ventricular muscle cells. This electrical conduction is due to the presence of gap junctions connecting the myocardial cells allowing electrical signals to travel from one to another. The sequential depolarization of all these cells determines the heart beat. Defects in the function of these cells may alter heart function.

The electrical activity of individual cardiac cells is presented as the myocardial action potential; which is divided into five distinct phases, due to the sequential opening and closing of different ion channels (Gralinski, 2000). The resting potential has a
value of -90 mV, see review by (ten Eick, 1992), which is close to the equilibrium potential for $K^+$. This is maintained by $K^+$ channels and $Na^+-K^+$ ATPase (see section 1.1.1). Since the resting membrane potential is very close to the reversal potential for $K^+$, it leaves the membrane highly sensitive to external $K^+$.

Myocardial cells found in different locations in the heart have very similar action potential phases with a difference in the duration (Figure 1-5). Exceptions are the sinoatrial node cells and atrioventricular cells, which transmit the signals over a very fast duration requiring mainly $K^+$ and $Na^+$ ions to maintain the resting potential and $Ca^{2+}$ to depolarize the cells. The five distinct phases of myocardial action potential are known as phase 0, 1, 2, 3 and 4 (see Figure 1-4).
Figure 1-5: Myocardial action potential waveforms.

The numbers indicate location of action potential. The arrows indicate the order of current directions during a heart beat. (Barry, 1996).
The resting phase of the action potential is known as phase 4. Depolarization of the cells caused by a change in the resting membrane potential towards positive potentials initiates phase 0. Phase 0 of the ventricular action potential is caused by the opening of the voltage-gated Na\(^+\) channels which increase the influx of Na\(^+\) ions (Figure 1-4). This decreases the membrane potential causing the initiation of the action potential. The Na\(^+\) channels responsible for the upstroke of the action potential in the heart are the Na\(_v\)1.5 channels (see section 1.1.2.1) encoded by the SCN5A-H1 gene (Balser, 2001). This isoform is highly expressed in the Purkinje fibres and is resistant to the puffer fish poison tetrodotoxin (TTX) (Ogata, 2002). The channel has a threshold of -60 mV, the \(V_{1/2}\) of steady-state inactivation is \(~-75\) mV (Fozzard, 1996) and fully inactivates at +20 mV.

The very rapid and intense influx of Na\(^+\) is quickly followed by inactivation of the Na\(^+\) channels (Gralinski, 2000), leading to the termination of the upstroke of the action potential in phase 1 (Figure 1-4) and an early phase of rapid repolarisation occurs due to the opening of the transient outward current (Crumb, 1999). This transient current is due to the activation of the voltage-gated K\(^+\) channels known as Kv1.2, 1.4, 1.5, 2.1/4.2 (Roden, 1996). These channels are abundant in the atrium, purkinje fibres, epicardium but not the sinoatrial node or the atrioventricular node. Activation and inactivation values vary among species and the K\(^+\) channels are inhibited by millimolar concentrations of 4-aminopyridine (4-AP) (Xu, 1998).

During phase 2, also known as plateau (Figure 1-4), there is an ionic balance between the inward depolarizing Ca\(^{2+}\) current through L-type Ca\(^{2+}\) channels and the outward
repolarization K⁺ current through the delayed rectifier \( I_{Kr} \) (Hiraoka, 1989). The change in membrane potential during depolarization causes the activation of the voltage-gated Ca²⁺ channels (L-type) as the Na⁺ channels become inactive. The activation of Ca²⁺ channels allows the entry of Ca²⁺ ions into the cell interior, which causes the release of Ca²⁺ from other compartments leading to muscle contraction and resulting in the heartbeat. These Ca²⁺ channels are responsible for the plateau and have a threshold of -25 mV and display small and slow inactivation (see section 1.1.2.2).

The repolarisation phase occurs, when the Ca²⁺ channels become inactive and several distinct K⁺ channels activate contributing to phase 3 of the action potential (Figure 1-4). This is caused by the two delayed rectifiers, the slow delayed rectifier (\( I_{ks} \)) and the rapid delayed rectifier (\( I_{Kr} \)), see reviews by (Nerbonne, 2000; Roepke, 2006). While \( I_{Kr} \) is encoded by the HERG gene and shows activation and inactivation properties, \( I_{ks} \) is encoded by mink and Kv.LQT1 and shows activation properties only. These delayed rectifiers terminate the action potential plateau and are present in all cardiac tissues.

The final phase of the cardiac action potential represents the maintenance of the negative resting potential of cardiac cells and is known as phase 4 (Figure 1-4). This is set by the inward rectifiers encoded by GIRK1 K⁺ family known as Kir2.1 and Kir3.1 (Gaborit, 2005). These channels are present in the atrium, purkinje fibres and highly expressed in the ventricles. They display no activation or inactivation properties and set the negative resting potential in response to the Na⁺/K⁺-pump.
1.2 The history of the electrocardiogram

The previous section concentrated on cellular action potentials at an individual cell level but the heart is composed of multiple cells which have an integrated electrical activity. In 1780, Italian anatomist Galvani noted a twitch in isolated frog legs when touched by metals. He referred to this observation as “animal electricity”, see (Cambridge, 1977). He later discovered that electrical stimulation of a frog’s heart resulted in cardiac muscle contraction. Nearly a century later, Waller published the first human electrocardiogram, which was recorded with a capillary electrometer (Waller, 1887). This publication led to the development of accurate recordings of electrocardiograms and its use as a clinical tool, see review by (Fye, 1994; Kar, 2005). The electrocardiogram has been used since as a medical test to detect cardiac abnormalities by measuring the electrical activity generated by the heart as it contracts.

In cardiology, the electrical waves of the whole heart are presented on an electrocardiogram (ECG) tracing. The ECG tracing represents the summation of cellular action potentials at the whole organ level, unlike the action potential which reflects the changes in myocardial membrane potential at the single-cell level, see review by (Elming, 2003). The individual waves of integrated electrical activity, which make up a single heartbeat, are called P, Q, R, S and T (Figure 1-6). The P wave represents the wave of depolarization spreading from the sinoatrial node throughout the atria. The atrial repolarization is not visible on the ECG as its amplitude is relatively small compared to the ventricular depolarization. The PR
wave is a representation of time between the onset of atrial depolarization and the onset of ventricular depolarization. The QT interval is measured from the beginning of the Q wave to the end of the T wave and therefore encompasses the entire period of electrical activation and recovery for the ventricles of the heart; the QRS complex is ventricular depolarization and the T wave is the repolarization phase. The PR interval occurs over 120 - 200 milliseconds and the QRS complex occurs over 80 - 120 milliseconds, any alterations to the heart rate will only affect the QT interval, where shortening of the QT interval indicates an increase in the heart rate.
Figure 1-6: ECG tracing.

The trace represents a single heartbeat which summarizes the electrical activity of the whole organ. The P-wave represents the activation of the atrium. The QRS complex summarizes the activation of the ventricles and the T wave represents ventricular recovery from activation. The QT interval represents ventricular activation and recovery.
1.3 Diseases affecting cardiac rhythms

As discussed earlier (see section 1.1.4), cardiac function is dependent on its regular contraction caused by electrical activity by the myocyte cells. Any alterations or dysfunction in any group of myocyte cells may lead to the development of arrhythmias and may lead to cardiac sudden death (Kannel, 1987; Willich, 1987). These arrhythmias can arise independently or as a result of an existing condition such as coronary artery disease or myocardial infarction (Hoffman, 1999). Heart rhythm disorders occur due to ectopic foci or malfunction of the heart conduction system, see review by (Zipes, 2003). Heart arrhythmias occur when signals from the sinoatrial node are not well-conducted in the heart or when a heart beat is generated from ectopic pacemakers, see review by (Viswanathan, 2006). These ectopic pacemakers generate potentials that are slow and are normally overridden by the fast sinoatrial node potential in a functional heart. The potentials generated by the ectopic pacemakers are only effective when potentials from the sinoatrial node do not reach ventricular cells in a diseased heart, see review by (Beery, 2005). These ectopic potentials do not usually follow the normal conduction pathways of the heart. This results in tachycardia or bradycardia depending on the location of the ectopic pacemaker, see review by (Yusuf, 2005).

Conduction abnormalities are caused by a delay in the transmission of impulses travelling from the atria to the ventricles. These abnormalities include conduction malfunction such as atrial fibrillation, ventricular fibrillation, premature ventricular contraction and heart block. Changes in the conduction system leading to arrhythmias are detected on an ECG.
One syndrome found to cause changes in heart function is the prolongation in the ventricular repolarization duration known as the long QT syndrome (Towbin, 2001b). The long QT syndrome (LQTS) is an inherited or acquired disorder causing prolongation in the QT interval on the surface of the electrocardiogram (ECG) (Chiang, 2000; Khan, 2002; Towbin, 2001b). The disease is characterized by syncope (fainting), resulting from episodes of atypical polymorphic ventricular tachycardia known as torsade de pointes, and affected individuals are at high risk of cardiac sudden death (Cheng, 2003).

The duration of the QT interval of the surface electrocardiogram (ECG), representing ventricular repolarizing duration, is determined by a balance between a number of inward and outward ionic currents (Shah, 2002a). It is prolonged usually when there is delayed repolarisation due to diminished outward K⁺ current during phase 2 and 3 of the action potential (Shah, 2002a). The prolongation in the QT interval (corrected QT) is diagnosed in females by an interval > 460 ms and in males > 440 ms (Taylor, 2003) compared to the normal interval values of < 430 ms for males and < 450 ms in females. Acquired LQT is caused unintentionally by anti-arrhythmic or by non-cardiovascular medication.
1.3.1 Congenital long QT syndrome (LQTS)

There are three forms of the inherited long QT disorder that have been described thus far which include the Romano-Ward (RW) syndrome, the Jervell and Lange-Nielsen (JLN) syndrome and sporadic cases (Towbin, 2001a). The two inherited forms have been characterized on the basis of the pattern of inheritance of the disorder; RW syndrome is a common (~99 % of inherited cases) (Vincent, 2002) autosomal dominant form with a pure cardiac phenotype. The JLN syndrome is a rare (1 % of inherited cases) (Vincent, 2002) autosomal recessive form characterized by the presence of cardiac abnormalities and inherited deafness (Priori et al 1999). JLN accounts for 1 to 1.6 cases per million in a population, estimated by Fraser et al (Fraser, 1964). The frequency of inherited LQTS in the USA is estimated at about 1 : 7000 people, resulting in an approximate 2000 - 3000 sudden deaths in children and young adults each year (Vincent, 2002). The inherited LQTS is caused by mutations in cardiac ion channels genes where five loci have been associated with the RW LQTS and are located on chromosomes 3, 4, 7, 11 and 21 (Jiang, 1994b; Priori, 1999; Schott, 1995; Wang, 1996c). These mutations are known as LQT 1 - 6 numbered by the time of discovery, where two loci are found on chromosome 21. Patients characterized as the sporadic cases do not display any genetic inheritance in family members. Their family members do not display any genetic indication of LQTS and thus patients are the first family members with the syndrome, carrying a mutated gene and displaying same clinical symptoms as the two inherited forms but usually without being deaf (Moss, 1991; Towbin, 2001a).
Congenital long QT syndromes 1 - 6 have been linked to mutations in specific proteins which form ion channels. The LQT1 is caused by a mutated KCNQ1 gene encoding the α-subunit of the I\textsubscript{Ks} channel protein (chromosomal locus 11p15.5) (Splawski, 1998; Wang, 1996b). This channel is responsible for the slow delayed current which plays a critical role in the repolarization phase of the ventricular action potential along with the rapid delayed current carried by the HERG channel; mutations causing loss of function inhibit K\textsuperscript{+} efflux and cause a delay in ventricular recovery from depolarization. The channel protein has 676 amino acids (Splawski, 1998) and more than 78 mutations have been found in LQT patients (Vincent, 2002).

A similar phenotype of the LQT1 syndrome is known as LQT5, caused by KCNE1 encoding Mink, an ancillary subunit for the KCNQ encoding I\textsubscript{Ks} channel complex (chromosomal locus 21p22.1-22.2) (Takumi, 1988). The protein is the β subunit which forms a tetramer with the α-subunit of the KCNQ potassium channel (Barhanin, 1996). Mutations found in this ancillary protein cause loss of function and therefore reduce K\textsuperscript{+} efflux as described with the mutant KCNQ1. The protein only has 129 amino acids and a few mutations have been reported (Russell, 1996).

LQT2 is caused by a mutation in the HERG gene encoding the rapid delayed rectifier K\textsuperscript{+} channel protein (chromosomal locus 7q35-36) (Jiang, 1994a). The loss of function mutations have similar phenotype to those mentioned for LQT 1 and LQT 5, as the HERG gene encodes for the α-subunit of the K\textsuperscript{+} channel partially responsible for the repolarization phase (Sanguinetti, 1996). HERG encodes for a protein of 1159 amino acids and more than 200 mutations have been identified (www.fsm.it/cardmoc/
The accessory subunit of the channel, KCNE2, is the cause of LQT6 (chromosomal locus 21q22.1) (Abbott, 1999). A few mutations in the protein lead to reduction in the outward current required for the maintenance of the repolarization phase of the cardiac action potential.

Another inherited form of the LQT is known as LQT3. This syndrome is caused by a mutation in SCN5A encoding the cardiac Na$_v$1.5 channel protein (chromosomal 3p21-24) (Wang, 1995). Mutations in this protein cause a gain of function unlike those identified earlier. Mutations in the channel inhibit the channel’s ability to inactivate which lead to constant flow of positive ions into the cells, keeping it in the depolarized state (Dumaine, 1996). This delays the repolarization phase which results in a prolongation in the duration of the action potential. This Na$^+$ channel encodes for a protein of 2016 amino acids, containing a transmembrane domain that acts as a voltage sensor.

The gene at chromosome 4 locus is caused by a mutation in ankyrin B, this form of the syndrome is known as LQT4 (Mohler, 2003). LQT1 is the most common form of inherited LQT syndrome, constituting 46% of cases, while LQT2 is the second most prevalent form of inherited LQTS, constituting 40% of all cases (Cheng, 2003).

### 1.3.2 Acquired long QT syndrome

The second cause of QT prolongation is known as acquired long QT syndrome. This form of the syndrome is not inherited and can be caused by several factors including electrolyte imbalance such as hypokalemia or hypomagnesemia (Curry, 1976;
Mineoi, 1992; Vincent, 2002), it can be caused by existing diseases such as neurological disorders and cardiomyopathies (Ryerson, 2006; Towbin 2004); as well as by medication. Prolongation of the QT interval can lead to the development of a life-threatening risk factor known as torsade de pointes (TdP), which has been frequently caused by drug intake (Elming, 2003).

Antiarrhythmic drugs such as dofetilide and sotalol were designed to correct irregular heart beats. Class III anti-arrhythmic drugs were intentionally designed to correct shortened QT intervals by inhibiting specific ion channels leading to a prolongation in the interval; however prolongation becomes toxic when it is excessive (Shah, 2002b).

### 1.3.2.1 Acquired long QT Syndrome caused by non-cardiac drugs

Non-cardiac drugs cause an unintentional prolonged QT interval and include anti-microbial agents, anti-fungal agents, antihistamines, tricyclic antidepressants and many more (Table 1-2).

Non-cardiac drugs may cause a change in the duration of the QT interval due to supratherapeutic plasma levels caused by an overdose or drug interactions with agents that inhibit their metabolism such as inhibition in the cytochrome P450 systems (Albengres, 1998). It has also been suggested that drug induced LQTS mainly occurs in patients with cardiovascular disease compared in patients that have no cardiovascular disease (Vieweg WV, 2002). Drugs have been found to unintentionally cause prolongation in the QT interval by inhibiting the HERG K+
channel, encoding the delayed rectifier current (Drici, 2000a; Eckardt, 1998; Krahenbuhl, 1995; Roy, 1996). Inhibition of the channel delays the outward current responsible for the repolarization phase in cardiac cells, leading to a defect in the heart rhythm. Inhibition of HERG has been the most frequent cause and so this is the main focus of the study.
Table 1-2: Example of the range of drugs causing Torsade de pointes (TdP) and prolong QT

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Class</th>
<th>Risks of TdP reported</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone</td>
<td>Anti-arrhythmic</td>
<td>+</td>
<td>(Torres, 1986)</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>Psychiatric</td>
<td>+</td>
<td>(Vieweg, 2004a)</td>
</tr>
<tr>
<td>Arsenic trioxide</td>
<td>Antineoplastics</td>
<td>+</td>
<td>(Ficker, 2004)</td>
</tr>
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<td>Astemizole</td>
<td>Anti-histamine</td>
<td>+</td>
<td>(Woosley, 1996)</td>
</tr>
<tr>
<td>Bepridil</td>
<td>Cardiac drug</td>
<td>+</td>
<td>(Campbell, 1990)</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Psychiatric</td>
<td>+</td>
<td>(Elkayam, 1980)</td>
</tr>
<tr>
<td>Cisapride</td>
<td>GI stimulant/heartburn</td>
<td>+</td>
<td>(Wysowski, 1996b)</td>
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<td>Clarithromycin</td>
<td>Antibiotic</td>
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<td>(Kamochi, 1999)</td>
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<td>+</td>
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<td>Doxein</td>
<td>Psychiatric</td>
<td>+</td>
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<td>Erythromycin</td>
<td>Antibiotic</td>
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<td>Gatifloxacin</td>
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<td>Grepafloxacin</td>
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<td>Category</td>
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</tbody>
</table>
1.4 Drugs causing QT prolongation by inhibiting the HERG K⁺ channel

1.4.1 Anti-arrhythmic drugs and their mode of action

Antiarrhythmic drugs are designed to correct irregular heart beats. These drugs are divided into several classes depending on their targeted membrane receptor or ion channel, see review by (Scholz, 1994). Antiarrhythmics acting on Na⁺ ion channels are classified as Class IA, Class IB and Class IC and include quinidine, procainamide and disopyramide; lidocaine, tocainide and mexiletine; encainide and flecainide respectively, see review by (Jaillon, 1989). Drugs such as propranolol and sotalol belong to the Class II antiarrhythmics blocking β-adrenoreceptors, see review by (Singh, 1987; Upward, 1988). Ca²⁺ channel blockers are known as Class IV and these include verapamil and diltiazem, see review by (Frumin, 1989). Class III antiarrhythmics prolong the repolarization phase and include sematilide, WAY-123,398, E-4031, RP 58866, dofetilide, ibutilide, amiodarone and the β-adrenoreceptor blocker sotalol, see reviews by (Doggrell, 2005; Dorian, 2000; Lombardi, 2006; Zicha, 2006). The Class III antiarrhythmics increase action potential duration without affecting conduction velocity by selectively blocking K⁺ (and not Na⁺) channels, see review by (Dorian, 2000; Nappi, 1993). These are used clinically to decrease the development of tachyarrhythmias, see review by (Daubert, 1993). Sotalol and amiodarone are used in the Australian market and have the ability to alter cardiac function by multiple mechanisms. Sotalol is both a non-selective β-adrenergic receptor antagonist and blocks delayed rectifier K⁺ channels, see review by (Singh, 1987). Whilst amiodarone exhibits a complex mechanism of action as it blocks Na⁺, Ca²⁺, and K⁺ channels and inhibits non-competitively α- and β-adrenergic receptors (Gonzalez, 1998; Tamargo, 2000). The incidence of torsade de pointes for sotalol is 2.4
% This increased to 5% at high doses in patients with impaired renal function and in the elderly (Dumas, 1989; Johnsson, 1976; MacNeil, 1993). Amiodarone blocks the HERG K+ channel leading to marked QT prolongation (Kiehn, 1999). In comparison to other class III antiarrhythmic agents, amiodarone suppresses early after-depolarisation in isolated cardiac preparations, due to its additional inhibitory effect on Ca2+ channel and β-adrenoreceptors (Tamargo, 2000).

1.4.2 Non-cardiac HERG blockers and their mode of action

Non-cardiac agents such as several antihistamines including astemizole, diphenhydramine, hydroxyzine, and terfenadine induce cardiac arrhythmia and torsade de pointes (Khan, 2002). These drugs prolong the QT interval, particularly in patients with severe hepatic diseases, hypokalemia or co-treated with drugs that inhibit metabolism pathways such as cytochrome P450 pathways, see review by (Tamargo, 2000). The cytochrome P450 pathway is required for the metabolism of parent drugs, the inhibition of the pathway leads to the accumulation of parent drugs in the plasma leading to toxic side effects. These drugs prolong the QT interval by blocking the cardiac K+ channel encoded by the HERG gene.

Several cases of QT prolongation, torsade de pointes and sudden cardiac deaths due to administration of antipsychotic and antidepressant drugs have also been reported (Teschemacher, 1999). Drugs such as amitriptyline (Teschemacher, 1999), doxepin (Strasberg, 1982a), imipramine (Wilens, 1996), haloperidol (Kriwisky, 1990), sertindole (Zimbroff, 1997) and pimozide (Krahentuhl, 1995) have been found to unintentionally cause widening of the QRS complex due to blockade of Na+ channels.
as well as the HERG K⁺ channel (Tamargo, 1992). Antipsychotics differ widely in their chemical structure and pharmacology and it is unlikely that all antipsychotics have the same effects on myocardial ion channels or on QT interval, or have similar pro-dysrhythmic potential (Taylor, 2003).

1.5 Expression and function of the HERG K⁺ channel

The HERG gene encodes the α-subunit of a delayed rectifier K⁺ channel along with MiRP1, a modulatory β subunit (Abbott, 1999; Weerapura, 2002). The ion channel is responsible for IKr, the delayed rapid rectifier responsible in part for action potential repolarization (with IKs and IKur) in cardiac myocytes as well as some neurons (see section 1.5). The channel displays features of shaker-type voltage-gated channels containing a voltage sensor, activation gate and a selectivity filter (Vandenberg, 2001). The HERG K⁺ channels belong to a family of voltage-gated K⁺ channels known as the EAG K⁺ channels (see section 1.1.2.3). The EAG channels are distinguished from other K⁺ channels by their six-membrane spanning domains (S1-S6), containing an EAG domain in the N-terminus and a cyclic nucleotide-binding domain (cNBD) in the C-terminus (Figure 1-7). The EAG domain is characterized by its first 135 amino acids of its N-terminus. This domain functions by binding to the S4-S5 linker controlling the time course of deactivation (Schwarz, 2004). Different homologs of the HERG channel have been found expressed in species such as rat, mouse, canine, rabbit and zebra fish (Langheinrich, 2003). The main site of variability in the amino acid sequence across these species is the extracellular loop of the S1-S2 transmembrane domains (Wymore, 1997), whilst the S4 voltage sensing domain is highly conserved across all homologs. Erg protein in canine and rabbit has 99%
identity to that in human, whilst rat and guineapig protein has 96% identity (Wymore, 1997; Zehelein, 2001). Studies completed to date on ERG currents in species such as zebrafish embryos have shown arrhythmias caused by a sensitivity to different classes of drugs (Langheinrich, 2003) as predicted in HERG including antihistamines, some psychotropic antagonists, gastrointestinal prokinetic drugs and antiarrhythmics (see section 1.4); drugs that are known to have no effect on the HERG K⁺ channel were also tested and have shown a negative effect (Langheinrich, 2003). The HERG gene is expressed in several tissues in human including smooth muscle (Farrelly, 2003; Shoeb, 2003), neurons (Farrelly, 2003; Warmke, 1994), cardiac tissue (Bertaso, 2002; Jones, 2004; Wymore, 1997) and in cancer tumour cells (Arcangeli, 2005; Bauer, 2003; Crociani, 2003; Lastraioli E, 2004; Pillozzi, 2002). Therefore, drugs can cause several lethal side effects including central nervous system depression, coronary artery spasm and seizures leading to sudden death, however, arrhythmias have been the most common (Tamargo, 2000).

HERG activation occurs after the initiation of a series of conformational changes within the protein (Jiang, 2005; Jiang, 2003). The cycles of conformational changes of the channel are characterized functionally by slow activation following depolarization and slow deactivation during hyperpolarization. However rapid voltage dependent inactivation and recovery from inactivation also occurs, see reviews by (Mitcheson, 1999; Tseng, 2001) (Figure 1-9). The channel displays inward rectification due to rapid-voltage dependent inactivation (Schonherr, 1996; Smith, 1996; Spector, 1996; Trudeau, 1995) causing a reduction in the amplitude of outward channel current (Spector, 1996). This is exceptional among mammalian voltage-gated
K⁺ channels. The channel is composed of 6 membrane-spanning domains (S1-S-6) and has a K⁺-selective pore between S5 and S6 (Trudeau, 1995; Warmke, 1994). The HERG K⁺ channel is classified as a voltage gated K⁺ channel due to the presence of the voltage sensor in transmembrane 4 (S4) which is composed of positively charged aligned amino acids (Trudeau, 1995) (Figure 1-7). This voltage sensor detects changes in membrane potential and causes conformational changes of the channel leading to its activation. The HERG encoded protein contains a eukaryotic PAS (Per-Arnt-Sim) domain at the N-terminus (Morais Cabral, 1998). The PAS domain is frequently involved in signal transduction and plays a role in the slow deactivation of the channel (Chen, 1999). The HERG protein contains a unique proximal domain that stretches from position 135 to 366 of its amino acid sequence. The domain contributes to the regulation of the channel gating (Gomez-Varela, 2002). The proximal domain also contains small sequences required for hormonal regulation of the channel’s gating (Gomez-Varela, 2003).
Figure 1-7: HERG K+ channel structure

A diagram representing the HERG K+ channel α-subunit. There are six transmembrane domains (S1-S6) and the selectivity filter is located between the S5 and S6 segments. The C-terminus contains a cyclic-nucleotide binding (cAMP) domain. Adapted from (Delisle, 2004).
Figure 1-8: Typical traces of whole cell recordings of HERG currents expressed in HEK293 cells (A), in Xenopus oocytes (B) and SH-SY5Y cells (C). Modified from (Bianchi, 1998; Taglialetela, 1998).
Figure 1-9: Kinetics of the HERG K⁺ channel.

HERG is unique in its fast inactivation and recovery from inactivation after depolarization and at the beginning of repolarization respectively. Panel A depicts a cardiac ventricular action potential. Panel B indicates the status of the channel at various time points in the action potential. Panel C shows a simplified diagram of the proposed conformational changes involved in channel gating. Diagram based on review by (Vandenberg, 2004).
1.5.1 Electrophysiology of HERG in transfected cell lines

The transfection of HERG into cultured cells has resulted in expression of an ion channel with biophysical and pharmacological properties close to, but not identical to the channel responsible for I_{Kr} in cardiac tissue (Zhou, 1998a). For example, the activation, deactivation and onset of block by methanesulfonanilides such as E-4031 and MK-499 in the I_{Kr} are slow (Wang, 1994); however, they are much slower for currents in HERG transfected systems. A study by Zhou et al (1998) on HERG K+ channels stably expressed in HEK293 cells reported that the kinetics of HERG encoded channels is dependent on temperature. Results obtained at 35 ºC were similar to results obtained in other expression systems including oocytes or in HERG transiently expressed in HEK293. The HERG channel activates at voltages positive to -50 mV but passes little outward current. Full activation of the channel is detected at a voltage of -20 mV. At positive voltages between 0 mV and +60 mV the channel displays inward rectification which has been linked to inactivation of the channel due depolarization of the cell (Sanguinetti, 1995). The half-maximum activation voltage for HERG was -4.2 mV at 23 ºC and -28.1 mV at 35 ºC in HERG transfected HEK293 cells (Zhou, 1998a). The study by Zhou et al (1998) suggested that HERG K+ channels behave similarly to native channels encoding I_{Kr} at physiological temperatures.

1.5.2 Drug binding characteristics on the HERG K+ channel

HERG inhibition by drugs can exhibit voltage-dependence and time-dependence as drugs may inhibit the channel by binding to either its activated or inactivated states. Potent HERG blockers such as MK-499 and dofetilide do not exhibit voltage-
dependence of inhibition. However, low affinity drugs such as quinidine do show voltage-dependence of inhibition (Sanchez-Chapula, 2001a). For example, inhibition of HERG by the methanesulfonanilide MK-499 occurs during activation of the channel and thus may be because the drug is trapped within the channel pore when the activation gate is shut (Mitcheson, 2000b). This trapping mechanism may explain the slow recovery of the channel from inhibition. This conclusion was supported by the inhibition of a mutant HERG channel characterized by its ability to open due to hyperpolarization (different to wild-type HERG) (Mitcheson, 2000b). This mutant channel was able to recover from inhibition within seconds, unlike the wild-type HERG channels which showed slow recovery to MK-499. These results suggest that HERG channels may have a large pore containing the binding site for drugs which may explain why large molecules can be trapped within.

1.5.3 Species response to $I_{Kr}$ inhibition

Many different species express different isoforms of the HERG K$^+$ channel (see section 1.5). This has made it difficult for researchers to determine the cardiotoxicity of drugs using animal models. Several animal-based testing systems have resulted in results variable to those obtained in human. To illustrate this, an example of a drug that produces different pharmacological effect in several systems compared to human is terfenadine.

1.5.3.1 Terfenadine

Terfenadine is a second-generation antihistamine designed in the early 1980s for the treatment of allergic rhinitis, urticaria and atopic dermatitis without sedative
properties (DuBuske, 1999). The first case reporting its cardiotoxicity was in 1989 (Davies, 1989); however it was not withdrawn from the market until 2000 when the death tolls were rising (Brown, 2004). It was reported that administration of terfenadine along with drugs such as ketoconazole which inhibit the cytochrome P450 oxidative pathways in the liver would lead to the development of torsade de pointes (Zimmermann, 1992). Terfenadine is metabolized by the cytochrome P450 pathway and inhibition of the pathway increases terfenadine levels in serum. It was later discovered that terfenadine had an inhibitory effect on the delayed rectifier current, $I_{Kr}$ (Delpon, 1999), which explained its cardiotoxicity. These reports led to the initiation of drug testing on $I_{Kr}$, where terfenadine was tested in different models to determine its lethal effects on cardiac cells. Reports on the inhibitory effect of terfenadine on different species have shown variability in the results including rat, guinea pig myocytes, dog purkinje fibre and heterologous cell lines (Table 1-3). The obtained half-maximum inhibitory concentration (IC$_{50}$) of terfenadine varied between 50 - 5960 nM in these tested systems (Carmeliet, 1998; Crumb, 2000; Ohtani, 1999; Suessbrich, 1996a; Tang, 2001). Rats are the most commonly used models in research and their use for testing the effect of terfenadine on cardiac cells have resulted in conflicting results compared to human. Terfenadine resulted in an IC$_{50}$ value of 6 µM in rat ventricular myocytes compared to that obtained in expression systems such as Xenopus oocytes containing the human HERG channel where 1µM of the drug completely suppressed the currents (Ducic, 1997). Terfenadine has been reported to cause little effect on the action potential duration in canine purkinje fibre at supratherapeutic concentrations (Gintant, 2001). It has also been reported that terfenadine has no effect on action potentials obtained from canine midmyocardial
fibres of left ventricular free wall, however it has been found to prolong the QT interval and cause Torsade de pointes in man (Zimmermann, 1992). Hence, a reliable model for testing the effect of drugs on cardiac tissue must be carefully selected (see section 2.2)
Table 1-3: IC$_{50}$ of terfenadine tested in various tissues

Summary of the inhibitory effect of terfenadine on different expression systems containing HERG or I$_{Kr}$. The half maximum concentration (IC$_{50}$) was determined using the voltage clamp technique.

<table>
<thead>
<tr>
<th>System</th>
<th>Potency IC$_{50}$ (nM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro-HERG transfected</td>
<td></td>
<td>Suessbrich, 1996a</td>
</tr>
<tr>
<td>Xenopus oocytes</td>
<td>246</td>
<td>1996a</td>
</tr>
<tr>
<td>HEK293</td>
<td>204</td>
<td>Crumb, 2000</td>
</tr>
<tr>
<td>CHO</td>
<td>56</td>
<td>Tang, 2001</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit I$_{Kr}$</td>
<td>96</td>
<td>Carmeliet, 1998</td>
</tr>
<tr>
<td>Cat I$_{Kr}$</td>
<td>180</td>
<td>Woosley, 1993</td>
</tr>
<tr>
<td>Guinea pig I$_{Kr}$</td>
<td>50</td>
<td>Salata, 1995a</td>
</tr>
<tr>
<td>Rat I$_{Kr}$</td>
<td>5960</td>
<td>Ohtani, 1999</td>
</tr>
</tbody>
</table>
1.6 Application of the 3Rs: Replacement, Reduction and Refinement

Animals are a useful tool in research for studying disease state and testing medicines prior to clinical testing. However, some species differ in their physiological function compared to human and studies conducted on them result in misleading conclusions (Weatherall, 1982) such with penicillin which was deactivated by blood when injected in rabbit, however it was later discovered beneficial to human, see review by (Ligon, 2004). Several societies have emerged to support researchers in applying animal welfare in their research including Dr Hadwen Trust, Americans Europeans Japanese for Medical Advancement, and the Australian Association for Humane Research.

In 1959 humane treatment of animals in research was initiated by applying the concept of the 3Rs: Replacement of animal procedures with non-animal procedures, Reduction in the numbers of animals used and Refinement of scientific techniques (Russell, 1959). In 2006, the European Union (Europa) issued a statement outlining an action programme designed to promote the concept of the 3Rs in partnerships between the European commission and industry sectors including pharmaceuticals, cosmetics and chemical manufactures. The programme was designed in order to support the development, validation and acceptance of alternative approaches in safety testing (Europa, 2006). This programme expands on the animal protection legislation that obliges industry sectors to apply the 3Rs to protect the welfare of animals as sentient beings (Europa, 2006). In fact, the European Union has already banned the testing of finished cosmetic products on animals in 2004 and a ban on animal testing of cosmetic ingredients and formulations will be initiated in 2009.
(Europa, 2003). This indicates that the issue with animal testing has become so critical that guidelines had to be set by regulatory bodies.

Animal testing is not only a regulatory issue but it has also become social where many animal-rights activists and medical professionals believe that it is inhumane, cruel and unethical. Supporters of animal testing claim that it is a requirement for medical progress. However, these testing methodologies can be replaced with alternatives that reduce or eliminate the use of animals in research. One process that has been successfully developed is the generation of antibodies in vitro. Antibodies were generated in animals by injection of molecules and the collection of animal tissue to isolate the antibodies. This has been replaced by the development of monoclonal antibodies which are generated using cells grown in the laboratory (Kohler, 1975). Hence, there are available alternatives to animal testing which may reduce or eliminate the need of animals in research. It also needs to be noted that animal research must be approved by animal ethics committees which aim at approving work that reduces animal suffering during experimentation and reduces the number of animals used in research (Committee, 2002). One cannot argue against the importance of animal testing in research at present, however animal suffering may be reduced if reliable in vitro assays are conducted to result in reliable data that would then help predict the number of animals required for testing in order to reduce their needs.

As discussed in an earlier section (section 1.4) the HERG K⁺ channel has been an unintentional target by various drugs, an effect not detected during preclinical
testing. This entire thesis focuses on the development of in vitro animal-free systems in drug testing to reduce the need of animal-origin reagents in research or drug testing.
1.7  Objective of the current study

1.7.1  Establishment and Validation of a HERG-Transfected HEK293 Cell Line Using Animal-Free Methods

Stably transfected cells lines are commonly used in various fields including cell regulation, protein expression and drug discovery testing. The aim of this study was to develop a transfection method using animal-free methodology for drug safety testing. This study involved stable transfection and cell culture using completely animal-free methodology. To validate the function of the inserted HERG gene, rubidium assays was developed as well as patch clamp electrophysiology.

1.7.2  The use of a neuroblastoma cell line to predict the inhibitory effect of drugs on HERG

Currently, preclinical testing of drugs effect on the HERG K+ channel has involved the use of HERG transfected cell lines. The aim of this study was to investigate the possibility of using an endogenous HERG expressing cell line for developing screening assays. The human neuroblastoma (SH-SY5Y) cell line expresses HERG K+ channels and was tested for its validity using animal-free methodology as a screening system using a rubidium assay.

1.7.3  Effect of Clomipramine on HERG K+ currents using different approaches for testing

Acquired long QT syndrome is a disorder caused by drug uptake. To date, several classes of non-cardiovascular drugs have been linked to long QT syndrome including antihistamines, antifungal, antimicrobial and psychotics. Clomipramine causes a prolongation in the QT interval, however, its mechanisms of inducing such effect has not been investigated. The aim of the current study was to determine clomipramine
effect on K$^+$ currents in HERG-transfected HEK293 cells using the voltage clamp technique. Clomipramine effect on HERG was also investigated in HERG-transfected HEK293 cells and SH-SY5Y cells using a rubidium assay.

1.7.4  PKC regulation of HERG currents in HERG transfected HEK293 cells

Protein kinases regulate the HERG K$^+$ channels by shifting the half activation voltage in Xenopus oocytes in the presence of the phorbol ester, PMA. The aim of this study was to determine whether the PKC specific activator, phorbol diacetate (PDA) has an effect on HERG K$^+$ currents in HERG transfected HEK293 cells using the voltage clamp technique.
Chapter 2 Establishment and Validation of a HERG-Transfected HEK293 Cell Line Using Animal-Free Methods.

2.1 Summary

1. A HEK293 cell line was stably transfected with the HERG $K^+$ encoding gene using animal-free methods.

2. Cells were cultured under animal-free conditions. The defined EXCELL-293 medium did not allow the cells to attach and grow in monolayer. Reagents which provided a healthy environment for cells included human serum, DMEM and human extracellular matrix. For transfection of the HERG plasmid, non-animal lipofectamine 2000 CD was used.

3. The stable transfection was successful, resulting in the formation of a cell line producing high levels of the HERG gene in comparison to native HEK293 cells.

4. Using specific HERG primers, the RNA in transfected cells was reverse-transcribed and amplified using PCR. Bands at an expected size of 575 bp in the transfected cells confirmed HERG presence in transfected cells in comparison to the native HEK293 cells.

5. The electrophysiological properties of HERG $K^+$ currents were examined in HERG transfected HEK293 cells using patch clamp electrophysiology. HERG activation was apparent at voltages positive to -40 mV and showed inward
rectification at voltages positive to -10 mV. The $V_{1/2}$ of the fitted activation curve of HERG tail currents was -23.0 mV ± 1.5. These currents were not seen in the native HEK293 cells.

6. A non-radioactive rubidium assay was developed to study the functional properties of HERG in the transfected cells. 50 mM K+ caused a significant increase in the rubidium efflux in comparison to the native HEK293 cell line. The efflux was inhibited by terfenadine, a potent HERG inhibitor.

7. K+ was tested at various concentrations and time intervals to determine the most suitable parameter for drug testing. 25 mM and 54 mM K+ caused a significant increase in rubidium efflux in comparison to 5.4 mM. 10 min of 54 mM K+ contact time with the cells also caused a rapid increase in efflux in comparison to 5.4 mM K+.

8. Validation of the system was completed using several HERG inhibitors. The IC$_{50}$ values obtained were compared to those obtained using electrophysiological studies in the literature. The correlation between these data and previously published values resulted in a correlation coefficient ($r^2$) of 0.76.

9. In conclusion, transfection of a human cell line using non-animal methods was successful and was validated using a non-radioactive rubidium assay. Results obtained were comparable to those available in the literature.
2.2 Introduction

Drug safety testing

All new chemical entities undergo safety testing before they are permitted to enter the clinical phase. One pre-clinical test that has become mandatory is the assessment of drugs’ effect on the heart and specifically on the QT interval which is a major cause of cardiac dysrhythmias (see section 1.3.2). Changes in the QT interval by drugs is caused by the inhibition of the rapid delayed rectifier current (I\textsubscript{Kr}) mediated by a K\textsuperscript{+} channel encoded by the HERG gene in humans (Drici, 2000b). The effect of drugs on this K\textsuperscript{+} channel has become a critical issue, since it has been found to be an unintentional target of non-cardiovascular medications, leading to the development of arrhythmias and in some patients has led to sudden cardiac death (Reynolds, 1976; Wysowski, 1996a). Terfenadine used for allergies (Roy, 1996) and cisapride used for the gastrointestinal system (Walker, 1999) were withdrawn from the market in 1998 and in 2000 respectively due to their risk of cardiac arrhythmias. Both drugs inhibit the HERG encoded K\textsuperscript{+} channel (see section 1.4 for more examples).

In 1997, the European Union (EU) issued a document titled “Points to consider: The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products” outlining the importance of determining the effect of medicinal products on the prolongation of the QT interval during the preclinical phase of drug safety testing. This document led to the issue of the ICH S7B guidelines in 2000 which lists recommended testing systems for new chemical entities. The guidelines aim to identify potential drugs of causing a change in the duration of the QT interval and to determine the toxic concentration of a drug and its metabolites. In \textit{vivo} and \textit{in}
*vitro* test systems have been recommended to eliminate the risks of patients being exposed to cardiotoxicity (Figure 2-1). There are issues with whole-animal testing due to differences compared to human. For example, animals express different ion channels to mediate the cardiac action potential compared to those expressed in human (see section 2.2.1.1) and this may not predict human QT prolongation.

The suggested non-clinical experiments required include: measurement of ionic currents in isolated mammalian cardiac myocytes, cultured cardiac myocytes or the use of heterologous expression systems for cloned human ion channels; action potential and ECG parameters on isolated cardiac tissue or on anaesthetized animals as well as pararrhythmic effects detected on isolated tissues or animals ((ICH), 2002). The various systems are described in the following sections.
Figure 2-1: Guidelines flowchart

Recommended non-clinical testing strategy for cardiac toxicity of new drug candidates. Adapted with changes from ICH S7B ((ICH), 2002).
2.2.1  *In vivo* methods

2.2.1.1  ECG parameters measured in animals

ECG recordings (see section 1.2) during pre-clinical testing can be done in both conscious and anaesthetized animals administered the test substance. Many factors can affect ECG recordings in animals including the choice of lead (single, chest or limb lead), animal status (conscious or anaesthetized), dosing period, measurement points, effect of heart beat on QT interval as well as the effect of the test substances on multiple sites in the heart (Guth, 2004). Common species that are frequently used for identifying drug cardiac toxicity include guinea pig, rabbit, monkey, ferret and dog (Bode, 2002). Test substances may act differently in animal models and might not necessarily provide a reliable estimate to human toxicity. Drugs such as loratidine and astemizole belong to the antihistamine class which have shown to have an inhibitory effect on human I_{Kr} encoded by HERG responsible for the QT interval (Crumb, 2000; Saviuc, 1993) but conflicting data was obtained in whole-animal experiments in guinea pig and rat (*Table 2-1*). These contradictions arose due to the difference in ionic currents underlying the cardiac action potential in some animals compared to humans (Nerbonne, 2004). Rats and mice do not provide a suitable testing tool as the current underlying the QT interval is the transient outward rectifier current I_{to} (Brunet, 2004; Josephson, 1984; Mitchell, 1984).
**Table 2-1: Effect of drugs on cardiac ionic currents mediated by K⁺ channels in dog, rabbit, rat and guinea pig.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Guinea pig</th>
<th>Dog</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Man HERG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astemizole</td>
<td>Antihistamine</td>
<td>Decreased Iᵦ&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Increased APD</td>
<td>Decreases Iᵦ&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Decreased HERG current</td>
<td>Decreased HERG current</td>
</tr>
<tr>
<td></td>
<td>(Salata, 1995b)</td>
<td>(Hashimoto, 1998)</td>
<td></td>
<td>(Carmeliet, 1992)</td>
<td>(Saviuc, 1993)</td>
<td></td>
</tr>
<tr>
<td>Terfenadine</td>
<td>Antihistamine</td>
<td>No change or increased APD</td>
<td>Minimal effect on APD</td>
<td>Decreased K⁺ currents</td>
<td>Decreases Iᵦ&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Decreased K⁺ currents</td>
</tr>
<tr>
<td>Loratadine</td>
<td>Antihistamine</td>
<td>No change K⁺ currents</td>
<td>No change K⁺ currents</td>
<td>No change in APD</td>
<td>No change in HERG</td>
<td>Decreased HERG current</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>Antiarrhythmic</td>
<td>Increase APD, Inhibits Iᵦ&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Increase in QT duration</td>
<td>Decreases Iᵦ&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Decreases HERG current</td>
<td>Decreased HERG current</td>
</tr>
</tbody>
</table>

Iᵦ<sub>K</sub>: rapid delayed rectifier, APD: action potential duration, QT interval: a time measurement on ECG reflecting ventricular activity, HERG: human ether-a-go-go-related gene
2.2.2 In vitro methods

2.2.2.1 Action potential parameters in isolated cardiac preparations

An action potential reflects the sequential activity of several ion channels within a cell. Assessing changes on the action potential allows for the identification of potential toxic effect of candidates on the heart. Since the ion channels responsible for each phase of the action potential have been previously identified (see section 1.1.4), changes in phase duration can help predict which ion channel is being inhibited by the test substances and so further protocols can be designed to investigate into individual ion channel function. For example, inhibition of the $I_{Kr}$ component is reflected on the action potential as a prolongation in the depolarization phase and this causes a slower rate of recovery from depolarisation at a whole heart level; which is reflected on the ECG tracing as prolongation of the QT interval. Since some drugs that prolong the QT interval do not change the duration of phases on the action potential (Gintant, 2001), both action potential studies as well as single ion current studies are usually considered (Martin, 2004).

Action potential recordings are done on isolated cardiac tissues such as purkinje fibers, papillary muscle, ventricular muscle and on isolated intact whole heart. The pharmacological studies done to date using such tissues have been completed from isolated animal tissues such as rabbit (Adamantidis, 1995; Dumotier, 2001; Eckardt, 1998; Puisieux, 1996), pig (Lacroix, 2000) and dog (Yan, 1998). The choice of tissue must be careful assessed as different parts of the heart express different channels responsible for the ionic currents and these may not necessarily participate in changes on the QT interval in man (Bode, 2002).
2.2.2 Ionic currents measured in isolated cardiac myocytes

The measurement of ion currents in native cardiac models provides an understanding of the natural regulation of ion channels in a cardiac tissue. However, the use of isolated cardiac myocytes is considered the most difficult approach as isolation of specific currents can be challenging. Many ion channels play a role in the regulation of the different phases of the action potential, such as phase 3 of the human ventricular action potential (see section 1.1.4) which is caused by two delayed currents, the slow and the rapid rectifiers. To isolate specific currents in a native cardiac cell, highly selective blockers of ion channels that might contribute to the current need to be selected. Techniques that allow for such testing include voltage clamp, which allows for the application of set voltages at which specific outward currents may be activated.

Animal and human cardiac myocytes are used as disaggregated cells in vitro and they may have altered ion channels, which provides great variability and misleading results (Bode, 2002). Human cardiac myocytes preparation availability is limited to atria or diseased ventricles, which may increase the variability, as channels in diseased ventricles could be defective. Further, the current encoded by the delayed rectifier channels is absent in the atria (Bertaso, 2002; Saint, 1999). Obtaining animal myocyte is an advantage over human as healthy cells can be isolated from healthy rabbit, dog or guinea pigs; however, it needs to be noted that ion channels responsible for the currents in these tissue may have different homology to those expressed in human (see section 1.5).
2.2.2.3 Measurement of ion currents in heterologous cell lines

The generation of continuous cell lines from mammals has yielded valuable tools for biological studies. Cell lines transfected with foreign genes are common in the development of high-throughput screening assays (Cheng, 2002; Tang, 2001; Terstappen, 1999). These have been favourable as they may contain only the protein of interest in high levels, which provide a more targeted approach. The use of mammalian cell lines for transfection such as human embryonic kidney cells (HEK 293) and Chinese hamster ovary cells (CHO) have been the most popular in drug safety testing as these cells do not express proteins that are critical in the normal function of the cardiovascular system (Cavero, 2000; Witchel, 2002; Zhou, 1998a; Zhu, 1998). Xenopus oocytes are widely used in studying the kinetics of ion channels at room temperature, where the ionic currents of ion channels can be detected 2 to 3 days after RNA injection of genes of interest (Witchel, 2002). The disadvantage of the system is that oocytes contain a large yolk in which lipophilic material interacts with test substances resulting in underestimated potency values (Witchel, 2002). It is also not possible to adapt the system as a high-throughput screening technology as oocyte testing can only be carried out using the two-electrode patch clamp technique. Mammalian cell lines are convenient as stable transfection provides consistent gene expression over periods of months (Zhou, 1998a), the cells can be stored away in liquid nitrogen and may still have the same expression levels as parental cells, and they can be adapted for high-throughput testing technologies such as fluorescence assays, flux and radiolabelled techniques (Netzer, 2001).
2.2.3 *In vitro* high-throughput screening assays

Many techniques have emerged for the measurements of the potency of drugs for cardiac safety testing (Finlayson, 2004). Detection of drug cardiotoxicity is now recommended in cell-based systems by the Committee for Proprietary Medicinal Products (CPMP). Many different types of testing methodology have been suggested for HERG encoded K⁺ channels including cell-based radioligand assays, fluorescence-based assays, flux assays and patch clamp (Bennett, 2003; Gill, 2003; Netzer, 2003; Netzer, 2001).

2.2.3.1 Radioligand binding assays

Radioligand binding assays were developed for the measurement of the affinity of drug binding to ion channels such as HERG K⁺ channel (Chiu, 2004; Finlayson, 2001b). The principle of the assay is that drugs binding to a common site within a channel to cause inhibition, therefore displacement of a radiolabelled drug may indicate HERG inhibitory effect. A potent inhibitor of the channel, such as dofetilide or astemizole are custom synthesized as [³H]-astemizole and [³H]-dofetilide in order to detect the potency of potential HERG K⁺ channel blockers (Chiu, 2004). The assay is based on the competitive displacement of the radio-labelled molecules by the test substance in intact cells or membrane assays (Chiu, 2004). The degree of displacement of the radioligand detected in the supernatant is a measure of the test substance affinity to the channel binding site. The assay is non-functional as it does not measure the effect of drugs on the ionic fluxes; it is only a measure of the binding parameters to the channel (Finlayson, 2001b). The assay only measures the binding affinity of a test substance competing over the same site as the radioligand. This is a
disadvantage, as drug binding to the ion channel is voltage-dependent (Carmeliet, 1992; Snyders, 1996a). The test relies on the single binding site hypothesis and test drugs might also be binding to other components in the membrane resulting in a high-false hits (Finlayson, 2001a). Chloroquine, a weak HERG inhibitor displays voltage-dependence of action (Sanchez-Chapula, 2001b). The drug blocks the channel when the channel is in the open state (Sanchez-Chapula, 2002). It has been proposed that the drug acts on two binding sites, a low and a high affinity site (Sanchez-Chapula, 2002). This indicates that HERG inhibitors may act on multiple binding sites with the channel’s pore.

2.2.3.2 Fluorescence assays

Fluorescent dyes have been in use for many years in the studies of ion movement across cells. In the past a few years, voltage-dependent dyes were developed to measure changes in membrane potential of cells. The DiBAC(4) dye is a voltage-dependent dye that has been used in drug discovery work as an indirect measurement of drug potency on ion channels. The dye principles are based on changes in the membrane potential value of cells caused by activation of channels. Changes in voltage-gated K+ channel activity such as HERG is associated with a change in the membrane potential (Netzer, 2001). Hence, inhibition of the HERG K+ channel by drugs can be detected by a change in membrane potential. The DiBAC(4) dye is negatively charged and is located in the extracellular solution under resting cell conditions. Depolarization of the cells moves the dye towards the cytoplasm where the potential is more positive (charge attraction) causing more fluorescence to occur. The disadvantages of this method are several including the slow kinetics of
the dye where dye displacement is slow allowing for measurements over minutes, drugs capability of quenching the dye (Tang, 2001), the fluorescence is influenced by foetal calf serum (Tang, 2001) and results are only an indication of membrane potential changes and not necessarily changes in individual ion channel function. These factors contribute to the probability of false-hit positives (Wolff, 2003). The fluorescent assays are carried in 96- to 384-well plates containing cultured cells where the fluorescence readout is not of a single cell, but is indicator of the activity of a whole group of cells. Thus, the system is not only indirect when measuring changes in the membrane potential but it is also an indicator of the activity of a group of cells.

The advantage of the assay is the development of high-throughput systems such as FLIPR (Fluorometric Imaging Plate Reader), which allows for automatic injection of solutions within the system and is capable of reading plates over 1 s, depending on the dye used and timing of pipette loading. The equipment allows for screening hundreds of substances a day.

A novel fluorescent based assay was developed for HERG testing known as HERG-Lite. The method is described as an antibody-based chemiluminescent assay. The technique predicts whether drugs inhibit HERG trafficking by acting as pharmacological chaperones or as inhibitors of hERG trafficking in two different cell lines that express HERG (Wible, 2005). The HERG channels are tagged with an HA epitope in the extracellular loop spanning the transmembrane domains S1 and S2. To date, this technique has predicted drug effect as blockers based on their ability to act as pharmacological chaperones or as trafficking inhibitors with no false positives or
negatives reported. However, the half maximal concentration predicted by the system to cause inhibition by drugs is underestimated by up to 100 fold compared to the gold-standard patch clamp values. This makes the system good for ranking drug potency but does not produce reliable estimates of drug toxicity.

2.2.3.3 Rubidium efflux assays

Radioactive ion flux assays have been in use for ion channel studies (Castelletti, 1989; Miller, 1990). These assays involve loading of cells with ions with similar characteristics to those transported through ion channels, such as K⁺ which can be mimicked by rubidium (Rb⁸⁶) (Hille, 1992). The assay depends on the ion channels ability to transport the loaded ion into the supernatant under applied stimuli, such as high K⁺ solution. The amount of efflux (supernatant) caused by the stimulus in the presence of the test substance is a direct functional measurement of the channels activity. However, it is not a highly desirable technique as it requires special handling requirements for radioactive Rb⁸⁶ and the amount of radioactive waste is a problem. In 1999, Terstappen (1999) developed a non-radioactive Rb⁺ assay, which involved the use of cold Rb⁺ as a tracer in place for K⁺ and the detection instrument used was atomic absorption spectroscopy. This technique is as reliable as the radioactive technique however its handling is safer and more high-throughput (Tang, 2001). This screening system also has disadvantages as it only allows for efflux measurement from a group of cells in well-plate formats. However, it has been adapted as a high-throughput screening assay and results to date have shown reliability in ranking the potency of drugs when compared to patch clamp (Chaudhary, 2006).
2.2.3.4  Patch clamp

Patch clamp is an electrophysiology technique used for the studies of ion channel function in excitatory tissues (Hille, 1992). The method has become useful in the study of ion channels in non-excitatory cells as well. The method is based on the application of voltage stimuli to cells and measurement of the direct current from ion channels within the membrane. In research, basic patch clamp equipment consists of microscopes with attached chambers connected to an amplifier which controls the applied voltages (controlled by software) and measures the output currents (Hille, 1992). The cells are cultured on coverslips placed in chambers containing electrophysiological solution. In whole-cell recordings, current is applied to cells via a fine glass micropipette containing a microelectrode soaked in a high salt solution which enhance currents through the studied ion channel within the membrane. These micropipettes are usually prepared from thin borosilicate glass tubes that are designed to have very fine tips with a diameter of a micron, which when in contact with the cell’s surface results in a seal of electrical resistance of giga-ohms. Whole-cell recordings occur when negative pressure is applied through the micropipette to rupture the cell membrane allowing for direct contact between the high salt solution in the micropipette and the cells intracellular compartment. This recording is a measure of all ion channels expressed in the entire membrane.

The patch clamp technique is considered the “gold-standard” in drug ion channel testing (Witchel, 2002) as it provides accurate information on the effect of drugs on ion channels such as state-dependency of inhibition, kinetics of channels and accurate potency values obtained directly from a single cell compared to a whole
group of cells. However, this technique is labour-intensive and low-throughput compared to the other drug testing systems as it requires highly skilled personnel (Finlayson, 2004). Time-limitations is another factor as cells don’t remain in their healthy state for longer than ~10 min due to the mechanical rupture caused by the micropipettes (Witchel, 2002). In the past few years, companies have been in the process of developing and launching robotic patch clamp equipments to facilitate the screening of a high number of substances in oocyte systems (Bennett, 2003). This equipment is very expensive and the reliability of the screen is compromised due the inaccuracy of using oocyte system (refer to section 2.2.2.3). Another high throughput patch clamp electrophysiology equipment is the planar patch clamp. The device consists of a planar chip that contains an aperture of micrometer dimensions (Fertig, 2002). Cells cultured in suspension are used where they are sealed onto the aperture by brief suction. This technique is high throughput as it eliminates the need for a microscope or a manipulator.

2.2.4 Drug structures

The wide range of drugs that inhibit the HERG K+ channel exhibit some structural similarities (Figure 2-2), which could predict a drug’s ability to inhibit the channel in structure activity modelling.

The use of chemical parameters of structures may also be useful to identify inhibitory effect on the channel. The development of computational methods for prediction of drug inhibitory effect is known as in silico screening (Dubus, 2006; Song, 2006b). These screening tools are developed by using experimental data from the literature.
Drugs with known IC$_{50}$ values are used to construct a relationship between observed and predicted data by obtaining their molecular descriptors such as hydrophobicity, charge and shape (Yoshida, 2006). Software generated from large databases provides an accurate prediction for drug toxicity and could therefore provide reliable estimations. Several predictive screens have been developed to date including two-dimensional (Yoshida, 2006) and three-dimensional quantitative structure-activity relationships (Pearlstein, 2003). Pharmacophore models were also developed which predict the drugs inhibitory effect on the basis of aromatic ring existence and its arrangement within the channels pore (Cavalli, 2002; Pearlstein, 2003; Song, 2006a). However, it has been observed that the effect of these drugs on HERG is state-dependent, which makes it difficult for one to predict a drug's effect. Hence, in silico screening can not replace laboratory testing as the current screening tools only predict chemical classes that might have a risk of inhibiting the HERG K$^+$ channel (Shah, 2005).

The aim of this study was to transfec a HEK293 cell line with the HERG K$^+$ channel gene using animal-free reagents and to use the cell line to develop a rubidium assay as a medium-throughput screening tool. This was completed to determine whether the elimination of animal components in all aspects of the system would produce a better prediction of drugs effect on the HERG K$^+$ channel. The success of the transfection was confirmed using animal-free techniques including PCR, patch clamp and a non-radioactive rubidium assay. These techniques were employed to study the biochemical as well as the functional characteristics of the HERG K$^+$ channels.
Figure 2-2: Structure of HERG inhibitors

Structures of the drugs tested on HERG K⁺ channel in this study. The inhibitors have a common motif, a single or multiple aromatic rings.
2.3 Methods

2.3.1 Transformation

2.3.1.1 Preparation of *Escherica coli* (*E.coli*) cells for electroporation

Competent *E.coli* DH5α strain (1 x 10⁹ cells/ ml) were thawed on ice for 5 min and aseptically inoculated onto Luria-broth (LB) agar plates (see 2.3.7) at 37 °C overnight. One evident colony was then picked and inoculated into 2 ml LB medium (see 2.3.7) and incubated at 37 °C overnight in an orbital shaker (Ratek Instruments, Melbourne, Australia) at 250 rpm. The cloudy preparation was then transferred into 500 ml of LB medium and incubated at 37 °C in an orbital shaker at 250 rpm. The Optical Density (O.D.) of the preparation was measured in a 1-cm plastic cuvette using a Hitachi spectrophotometer (colorimetric measurement) at 600 nm every 45 min until the O.D. reached 0.7 - 0.8 A₆₀₀ units/ml. This absorbance value is an indicative measurement of bacterial growth. Cell density readings between 1 and 1.5A₆₀₀ units/ml are equivalent to 1 x 10⁹ cells/ ml (Invitrogen Midi prep kit manufacturer’s protocol). Once the required O.D. was achieved, the preparation was placed on ice for 20 min. The preparation was then centrifuged at 5000 g at 4 °C for 10 min to separate the bacteria from the medium. The supernatant was discarded and the pellet was resuspended in ½ original volume glycerol in water (10 % v/v) and the mixture was thoroughly mixed by vortexing until all homogenate was re-suspended. Several wash steps were then performed as follows: the preparation was centrifuged at 5000 g for 10 min at 4 °C and the supernatant was discarded. The pellet was then re-suspended in ¼ original volume glycerol (10 % v/v), thoroughly mixed until all homogenate re-suspended and re-centrifuged as above. The supernatant was then
discarded and the pellet was re-suspended in 1/20 original volume glycerol (10 % v/v), thoroughly mixed until homogenate re-suspended and re-centrifuged as above. The supernatant was then discarded and the pellet was resuspended in 1ml glycerol (10 % v/v). The preparation (E.coli bacteria) was then aliquoted into sterile microfuge tubes at 50 μl/ tube. The E.coli cells were then stored at -80 °C until used.

2.3.1.2 Plasmid electroporation

The HERG1 plasmid containing the HERG DNA was obtained from Professor Arcangeli (Department of Experimental Pathology and Oncology, University of Firenze, Italy) cloned into the pcDNA3.1 vector between BamHI and HindIII sites (see Figure 2-3). The vector contains neomycin and ampicillin resistant genes. The plasmid (4.5 ng/μl) was electroporated into DH5α bacteria using Gene Pulser II Electroporation System (Bio-Rad Laboratories, CA, USA). The bacterial cells (prepared as in section 2.3.1.1) were incubated on ice for 20 min prior to electroporation to allow bacteria to thaw. The voltage on the electroporation apparatus was set on 2.5 kV at a low resistance range of 200 ohms and a high resistance range of 500 ohms; and a capacitor setting at 25 μF. These are the recommended settings by BIORAD for electroporating DNA into gram negative bacteria. Pre-cooled 0.2 cm gap cuvettes were used for the electroporation by adding 50 μl of bacteria (≥ 7 x 10⁸ cells/ml, O.D. 0.7 - 0.8) to the plasmid (9 ng). Once in the electroporation system and the appropriate pulse was applied to the cells as per settings, the bacteria were thoroughly mixed in 1 ml SOC medium (see 2.3.1.1) and placed in a 15 ml flat-bottom tube. The electroporated bacteria were placed in a 37 °C incubator for 1 h and shaken at 250 rpm. Then 100 μl of the preparation was spread
onto an LB agar plate containing ampicillin (100 μg/ ml) and incubated at 37 °C overnight. Ampicillin was added as the HERG1 plasmid vector expresses an ampicillin-resistant gene and therefore only the electroporated cells grow. Evident colonies were isolated and cultured into 40 ml of LB medium containing ampicillin (100 μg/ml) overnight. The plasmid was then purified out using a midi prep kit (Invitrogen, Carlsbad, USA), (see 2.3.1.3).
Figure 2-3: pcDNA3.1 vector map (Invitrogen Corporation).

The HERG gene was cloned between the Hind III and Bam H sites. The vector expresses a penicillin resistant gene, required for bacterial transformation. It also contains a neomycin resistant gene, which enables the selection of stable transfected cells that are expressing genes within the vector in the presence of neomycin.
2.3.1.3 Plasmid purification

The prepared plasmid (see section 2.3.1.2) was purified using a Midi prep kit (GIBCO Concert High Purity Plasmid Purification Systems, Invitrogen). The kit uses an anion exchange resin to purify plasmid DNA from bacterial suspensions. It involves several steps including washing of the column, binding of DNA to the resin and DNA elution. The kit was used as per manufacturer’s protocol as follows: 10 ml of equilibration buffer (see 2.3.7) was added to the purification column. The solution was allowed to drain through gravity and was discarded. 40 ml of the bacterial preparation transformed with HERG1 (see 2.3.1.2) was centrifuged at 15000 g and the supernatant was discarded. 4 ml of the cell suspension buffer (see 2.3.7) containing RNase A was added to the pellet and vortexed till the mixture was all homogeneous. The RNase A is added to digest RNA while preparing DNA samples. 4 ml of lysis buffer (see 2.3.7) was added and the solution was mixed gently by inversion, and incubated at room temperature for 5 min. This step is required to release DNA from the bacteria into the solution. The lysis buffer contains SDS which denatures the bacterial proteins; and also contains NaOH which denatures the chromosomal and plasmid DNA. 4 ml of neutralization buffer (see 2.3.7) was added and mixed by inversion to stop further activity of the lysis buffer. The neutralization buffer contains potassium acetate which causes covalently closed plasmid DNA to re-anneal rapidly; while the chromosomal DNA, bacterial proteins and SDS are precipitated by the potassium acetate, forming a complex with the potassium. The preparation was centrifuged at 15000 g at room temperature for 10 min to remove all unwanted formed complexes. The supernatant was added to the equilibrated column and the solution was allowed to drain by gravity flow. This step allows the
negatively charged phosphates on the backbone of the DNA to interact with the positive charges on the surface of the resin. The eluate was discarded and the column was washed twice with 10 ml of wash buffer to remove any unbound DNA. The eluate was discarded and the DNA in the column was eluted by adding elution buffer (see 2.3.7), elution is achieved under high salt conditions which separate the bound DNA from the column. The flow-through was collected and 3.5 ml isopropanol was added and the mixture was centrifuged at 15000 g at 4 °C for 30 min. This step is critical as it desalts the DNA and concentrates it using an alcohol precipitation. The supernatant was discarded and the pellet was washed in 3 ml of ethanol (70 %) and centrifuged at 15000 g at 4 °C for 5 min. The ethanol was discarded and the DNA pellet was allowed to air dry for 10 min. The DNA pellet was dissolved in 100 μl of distilled water.

The optical density (O.D.) of the DNA was measured in a 1 cm wide quartz cuvette using a UV spectrophotometer taking readings at two wavelengths 260 nm and 280 nm. The reading at 260 nm provides an estimate of the nucleic acids in the sample. The reading at 280 nm provides an estimate of other contaminants such as proteins and UV absorbers. Calculation of the 260 : 280 ratios is required to determine the level of contamination in the sample. If the ratio is 1.8 - 2.0, then the absorption is due to nucleic acid and indicates that the sample contains mainly DNA. If the ratio is < 1.8, it indicates that there are proteins or other UV absorbers in the sample. If the ratio is > 2.0, it indicates that the sample could be contaminated with chloroform or phenol. A fraction of the DNA was electrophoresed on a 0.7 % (w/v) agarose gel (Promega, Madison, USA) at 70 V for 60 min to confirm the DNA size in comparison
with lambda base pairs markers (Promega, Madison, USA). This step is required to confirm that the O.D. of the nucleic acid is due to the presence of the purified DNA. Hence the size is estimated by comparison to standard base pair markers.

2.3.1.4 Plasmid linearization

For stable transfection of the HERG1 plasmid, linearization of the plasmid was required to allow the DNA to be correctly inserted into the genome. The HERG plasmid restriction map and the vector restriction map were used to determine the restriction enzyme capable of digesting a single site in the expression vector. The restriction enzyme \textit{Pvu I} was used for plasmid linearization as it only cuts the ampicillin-resistant gene site in the expression vector as the ampicillin resistant gene was not required for transfection. 10 \( \mu l \) of plasmid (1.39 \( \mu g/ \mu l \)) was added to 16 \( \mu l \) 10x D buffer (see 2.3.7), 16 \( \mu l \) acetylated bovine serum albumin (1 mg/ml), 114 \( \mu l \) DNase and RNase-free water and 4 \( \mu l \) of \textit{Pvu I} (10 U/\mu l). The mixture was centrifuged briefly and incubated at 37 \(^\circ\)C overnight. A fraction of the preparation was loaded onto agarose gel (0.7 \% w/v) and electrophoresed at 70 V for 60 min to confirm plasmid digestion. The total size of the plasmid including the vector (5400 bp) and the HERG1 insert (3508 bp) was expected to be 8908 bp. Digestion was confirmed by the presence of a single band which represents linear DNA, compared to undigested plasmid which displays three bands on agarose representing three forms of the DNA: nicked, linear and supercoiled.
2.3.1.5  **Plasmid precipitation**

Plasmid precipitation was completed to remove all unwanted components of the digest mixture. The digested DNA solution was made up to 400 μl in DNase-free water. 400 μl of Phenol/chloroform (1 : 1 v/v) solution was added and the mixture was vortexed and centrifuged briefly at 14000 g. The upper layer was then removed into a clean microfuge and 400 μl of chloroform was added. The mixture was vortexed and centrifuged at 14000 g for 30 s. The upper layer was removed into a clean microfuge tube and the previous step was repeated. 40 μl of sodium acetate (3 M) and 1 ml of absolute ethanol was added and the DNA was allowed to precipitate on ice for 1 h. The mixture was vortexed, the supernatant was discarded and the pellet was suspended in 500 μl ethanol (70 % v/v) and centrifuged at 14000 g for 5 min. The supernatant was discarded and the pellet was allowed to air dry for 10 min. The pellet was resuspended in 20 μl DNase-free water and kept at -20 °C until used. A fraction was electrophoresed on agarose gel (0.7 % w/v); this was completed to confirm the presence of the correct DNA size.

2.3.1.6  **Plasmid sequence**

Prior to transfection of cells with the HERG plasmid, the plasmid was sequenced to confirm the correct HERG map. This was completed by providing Micromon DNA sequencing facility (Monash University, Melbourne) an aliquot of the final plasmid preparation. The sequencing was then completed by incubating the plasmid with the appropriate primer. For this plasmid the primer used for sequencing was the T7 primer, which identifies a region on the vector that is ~8 base pairs from the HERG
insert. This primer was selected as per vector manufacturer’s protocol (Invitrogen, Carlsbad, USA). The sequence for the T7 primer is:

Forward primer: 5’ TAA TAC GAC TCA CTA TAG GG3’
Reverse primer: 5’ GCT AGT TAT TGC TCA GCG G 3’

The resulting sequence should start with the above sequence and continue at the beginning of the HERG1 insert if correct.

2.3.2 Culture of human embryonic kidney cell line-HEK293

The human embryonic kidney (HEK293) cell line was obtained from American Type Culture Collection (ATCC, Manassas, USA). Cells were grown under various conditions to determine which provides the most optimal non-animal conditions.

Condition 1: A chemically-defined serum-free growth medium-EX-CELL™ 293 HEK 293 was tested. Calcium was added at a concentration of 0.012 M and L-glutamine at 0.006 M. Calcium was added to allow for cell adhesion and poly-l-lysine. Cells were maintained at 37 ºC in a 5 % CO2 sterile incubator.

Condition 2: Serum containing growth medium (human serum).

The cells were maintained in growth medium containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with human serum (10 % v/v) and gentamicin (15 µg/ml) at 37 ºC and 5 % CO2 sterile incubator. Cell detachment from polystyrene plastic ware (Nunc easy flasks, Medos Australia) was done by the
addition of TrypLE Select, a trypsin-like bacterial dissociation enzyme. Culture medium was exchanged in culture flasks every 2 days. Confluent cells were passaged by the addition of 1 ml TrypLE Select for 3 min at room temperature. Cells were then resuspended in 5 ml DMEM and spun at 100 g for 5 min at room temperature. The pellet was then resuspended in 5 ml of growth medium and 0.5 ml of the suspension was added to five 75 cm² culture flasks. Cells were stored in growth medium containing DMSO (10%) in liquid nitrogen.

2.3.3 Transfection of cells

HEK293 cells were cultured in growth medium as per condition 2 (see section 2.3.2) on human extracellular matrix (ECM) coated 75 cm² flasks for the transfection process. Human extracellular matrix (BD Biosciences, San Jose, USA) was prepared in DMEM at 0.1 mg/ml and kept at -80 °C until used. A 1:2 dilution of ECM was prepared prior to coating of flasks. 500 μl of the diluted ECM was aseptically added to each well in a 6-well plate using a sterile syringe and needle. The surface of the flask was completely covered with ECM and kept at room temperature (RT) for 2 h under sterile conditions in a tissue culture hood. The flasks were then washed twice with DMEM to remove any unbound ECM. Confluent cells (~80%) were subcultured onto a 6-well plate in growth medium 24 h prior to transfection. The cells were detached from the confluent flask by the addition of 2 ml TrypLE Select. 8 ml of growth medium was added and the cells were spun at 200 g for 5 min at RT. The cell pellet was resuspended in 6 ml growth medium and 600 μl of the suspension was added to each well containing 1.4 ml growth medium. Once the cells attached to the bottom of the wells after 16 h of culture, the medium was exchanged to gentamicin-
free medium 4 h prior to transfection as gentamicin causes cell death in the presence of the transfection reagent. On the day of transfection, 4 μg of linearized plasmid was incubated in 250 μl of Opti-MEM I (see 2.3.7) in a sterile microfuge tube, while 10 μl of the transfection reagent, lipofectamine 2000 CD (see 3.4.7) was incubated in 250 μl Opti-MEM I in another microfuge tube for 5min. Both preparations were then mixed together and incubated for 20 min at RT. The growth medium was exchanged to gentamicin-free medium containing reduced human serum concentration (2 % v/v). Serum may compromise transfection efficiency so it is a requirement to reduce its concentration. The plasmid-lipofectamine complex was added to the cells and the plates were rocked gently. 24 h post-transfection, cells were subcultured into another 6-well plate in normal growth medium; the cells were dissociated using TrypLE Select and resuspended in growth medium and further subcultured without centrifugation. Selective medium containing Geneticin (0.01 mg/ml) was added after 24 h to allow for cell attachment. Geneticin (G418) was used as the selection drug as the HERG plasmid’s vector contains a neomycin resistance gene, cells containing the plasmid will be resistant to Geneticin; however cells that were not transfected will be destroyed. The cells were cultured in selective medium containing Geneticin until colonies were evident. The selective medium was exchanged every 4 days, to prevent antibiotic break down. Once colonies were evident after 13 days, they were expanded into larger flasks and were cultured in selective medium for 14 days. The experiment contained several controls including: Control 1: cells incubated with Geneticin but no transfection, Control 2: cells incubated in growth medium, Control 3: cells incubated with transfection complex but no Geneticin.
### 2.3.4 Polymerase chain reaction (PCR)

The presence of HERG in transfected HEK293 cells was assessed by detecting RNA levels using polymerase chain reaction (PCR). Several steps were employed to complete the process as follows:

**RNA isolation:** RNA from confluent cells was collected using TRIZOL reagent as per manufacturer’s protocol (Invitrogen, Carlsbad, USA). TRIZOL is a monophasic solution of phenol and guanidine isothiocyanate used for RNA isolation from tissues, an improvement of a method previously developed by Chomczynski and Sacchi (Chomczynski, 1977). The reagent maintains RNA integrity while disrupting the cell membrane and dissolving cell components. Confluent cells in 75 cm² flasks were washed three times with ice-cold phosphate buffered saline (see 3.5.7). 5 ml of Trizol reagent was added to the flask and the cell lysate was passed through a 5 ml glass pipette and transferred to a 15 ml centrifuge tube. The lysed cells were then incubated at RT for 5 min to allow for complete dissociation of nucleoprotein complexes. 1 ml of chloroform was added and the samples were mixed vigorously for 15 s followed by incubation at RT for 3 min. The sample was centrifuged at 15000 g for 15 min at 4 °C. The centrifugation step causes the TRIZOL reagent to break into three layers to form, a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase contains all the RNA and was removed into a clean tube. The RNA in the aqueous phase was precipitated with 2.5 ml isopropyl alcohol and incubated at RT for 10 min followed by centrifugation at 12000 g for 10 min at 4 °C. Centrifugation led to the formation of a small gel-like pellet; this is the precipitated RNA. The supernatant was discarded and the pellet
was washed by vortexing with 2.5 ml of 75 % ethanol and centrifugation at 7500 g for 5 min at 4 °C. The supernatant was then discarded and the pellet was allowed to dry at RT, 50 μl of RNase-free water was added and the sample was incubated at 55 °C for 10 min. Samples were stored at -80 °C until used for PCR.

**Reverse transcription of RNA:** Isolated HERG RNA was reverse transcribed using Sensiscript master mix (Qiagen) containing 1 μl Sensiscript Reverse Transcriptase as the reverse transcriptase enzyme required for complementary DNA (cDNA) synthesis in the presence of Deoxynucleotide Triphosphates (dNTP) Mix (0.5 mM), 1x Buffer Reverse Transcriptase, HERG RNA (50 ng) and RNase-free water to a final volume of 25 μl. Samples were incubated at 37 °C for 60 min.

**PCR:** Reverse transcribed RNA (1 μg) was mixed with master mix containing: 1 x PCR buffer (see 2.3.7), 1 x Q-solution (see 2.3.7), dNTP mix (200 μM), primer sense erg1 (0.5 μM), primer anti-sense erg1 (0.5 μM), 2.5 units/reaction HotStarTaq DNA Polymerase and RNase free-water to a total volume of 25 μl.

The sequence of oligonucleotides primers was as follows:

- Primer sense erg1: 5’-TCCAGCGGCTGTACTCGGGC-3’
- Primer anti-sense erg1: 5’TGGACCAGAAGTGGTCGGAGAACTC-3’

The primers start at nucleotide 2171 and nucleotide 2746 of the HERG1 sequence (accession number U04270), respectively.
PCR was completed using a Perkin Elmer GeneAmp PCR System 2400. After 15 min of enzyme activation at 95 °C, 43 cycles of amplification was completed, these included denaturation at 94 °C for 1.5 min, annealing for 3 min at 55 °C and extension at 72 °C for 1.5 min (Figure 2-4). DNA products were electrophoresed on an agarose gel (0.7 % w/v) at 70 V for 60 min. A 1 Kb DNA ladder was used as a molecular weight marker (Invitrogen, Carlsbad, USA). Bands were detected by mixing the DNA products with cyber green and visualization using a UV transilluminator (DyNA Light UV transilluminator, Labnet International, Woodbridge, USA). Three samples were prepared under the same conditions: HERG-transfected HEK293, HEK293 and the HERG plasmid.
Chapter 2

Figure 2-4: PCR principles.

The double stranded DNA is first broken up to two single strands by denaturing at 94 °C for 1 min. The specifically designed primers are then allowed to attach to the single stranded DNA during the annealing phase which occurs at 54 °C for 3 min. The complementary strand of DNA is then synthesized in the presence of the specifically designed primers during the extension phase in the presence of Deoxynucleotide Triphosphates. The cycle is repeated 43 times to allow for detectable DNA concentrations.
2.3.5 Patch Clamp

2.3.5.1 Cell preparation

*Coverslip sterilization:* 25 mm² diameter round coverslips were placed in a 6-well plate. The coverslips were UV radiated in cell culture hood for ~1 h prior to cell addition.

*Subculture of cells:* HERG-transfected HEK293 cells were cultured in 25 mm² culture flasks in DMEM containing gentamicin and human serum (10%). One day prior to patch clamp experiments, cells were cultured onto sterile 25 mm² coverslips. Cells were detached from flasks by the addition of 1 ml of TrypLE Select for ~2 min and resuspended in 4 ml DMEM only (without serum). Cell suspension was centrifuged at 100 g for 5 min at room temperature; the cell pellet was then resuspended in 5 ml of growth medium (see section 2.3.2). Cell suspension (200 μl) was added to each coverslip in a 6-well plate for 10 min before 1.5 ml of growth medium was added and the plate was placed in 5 % CO₂ sterile incubator for ~16 h.

2.3.5.2 Preparation of patch pipettes

Patch pipettes were made out of thin-walled borosilicate fine glass capillary tubing with filaments pulled by a two-stage procedure in a pipette puller (Sutter P-97 flaming/brown micropipette puller, Sutter Instrument Company, CA, USA). The middle of the glass tube is first pulled a short distance resulting in thin tubing, followed by a second pull resulting in two pipettes with a relatively large-diameter tip. To be able to obtain the right diameter size of a pipette, puller parameters were
adjusted and several protocols were tested to obtain a pipette that has resistance of 2 - 4 Meg ohms.

2.3.5.3 Whole cell recording

On the day of experiments, cells on coverslips were placed in a heated recording chamber on an Olympus BX51 upright microscope (Olympus Corporation, Tokyo, Japan). The chamber was continuously superfused with external solution (see section 4.3.3) and the temperature was maintained at 37 °C using a temperature controller attached to the chamber (TC-1 temperature controller, Biosciences Tools, CA, USA). The membrane currents were recorded in whole-cell configuration-using patch electrodes. Whole cell currents were recorded using an Axopatch 200B patch clamp amplifier (Axon Instrument, Molecular Devices Corporation, CA, USA) and digitised using an Axon digitiser and Axon pClamp software (Axon Instrument, Molecular Devices Corporation, CA, USA). All data analysis was completed using Igor Pro (WaveMetrics, Inc., Oregon, USA).

To achieve a whole-cell recording, several steps were employed:

Solutions: Internal solution (see section 4.3.3) was prepared and aliquotted in microfuge tubes and kept at -20 °C until used.

Patch pipettes: The pipettes were prepared on the day of the experiments and were kept in a capped plastic container until used to avoid the entry of dust particles which may block the fine tip pipettes.
Patching a cell: Prior to patching onto a cell, a cell was picked using the high objective on the microscope. Round attached cells were chosen as they were easier to seal onto than flat cells. Once a cell was chosen, a patch pipette was filled with the internal solution (see section 4.3.3) using a 1 ml syringe attached to thin pipette filler, avoiding the formation of bubbles. Before the pipette was inserted into the bath containing the cells, pressure was applied via tubing connected to the pipette holder by blowing in air. This positive pressure causes the internal solution to flow out of the pipette preventing any dust particles from entering the pipette. Once the pipette is in the solution, the pipette voltage offset was adjusted to zero to compensate for any voltage offset in the circuit. The holding potential was set to -80 mV and switched to voltage clamp mode. The pipette was then brought closer to the cell membrane until it was in touch with it; the positive pressure was taken off the tubing while monitoring the resistance value. The resistance value was allowed to stabilize over a period of 2 min. The seal resistance value needed to be in the range of 5 – 50 giga ohms; this value indicated a good seal between the cell and the patch pipette. Once this seal resistance had stabilized, compensation of the electrode capacitance currents was applied whilst applying holding potential. A negative pressure pulse was then applied to the tubing to allow for membrane rupture and forming a direct contact between the cells interior and the pipettes internal solution. The success of the process is detected as an increase in the size of the capacitative transient on the digitized current waveforms and once this was achieved the cells was known to be in a whole-cell configuration. When tested, clomipramine was added to the external bathing solution.
Cells showing currents due to leak were eliminated from further analyses as this leak was due either to unhealthy cells or loss of contact between the cell and the pipette tip. These leak traces were distinguished by a loss of tail current recordings. Capacitance compensation was accounted for prior to recordings. The size of peak tail currents of traces were analysed as an indicator of channel activation. Tail currents were used since all recordings were carried at -50 mV, which minimised variability caused by leak.

2.3.6 Rubidium efflux assay

The rubidium assay was developed to test whether the transfected HERG channels were functional. Rubidium (Rb⁺) has properties like K⁺ and gets transported through K⁺ channels. Measurement of Rb⁺ efflux in cells is an indicator of channel transfection when compared to non-transfected cells. The cells were incubated with Rb⁺ (5.4 mM), which is the same physiological concentration as extracellular K⁺, to replace K⁺. Activation of the K⁺ channel leads to the opening of the channel and transport of Rb⁺ out of the cell.

2.3.6.1 Rb⁺ Assay conditions

2.3.6.1.1 Cell count

Prior to each experiment, cells were dissociated from culture flasks and a cell count and viability were determined using trypan blue exclusion (see 2.3.7). Trypan blue is a blue acid dye used for cell estimation in a population. Viable cells are distinguished from dead cells by their ability to take up the dye. Dead cells have damaged
membranes which allow the dye to be taken up, appearing blue. Once cells have been detached from culture flasks, they were suspended in serum-free DMEM. This suspension medium avoids the interference of binding of trypan blue to serum proteins. A small aliquot of the cell suspension was then mixed with trypan blue at a 1:1 ratio. 10 μl of the mixture was added to the hemocytometer and the number of viable cells was determined by counting cells in each square.

2.3.6.1.2 Rb⁺ efflux assay

HERG transfected HEK293 cells were cultured on poly-l-lysine coated 24-well cell culture micro-plates (BD Biosciences) for 18 h to a final density of 2 x 10⁵ cells/well. The growth medium was removed and the cells were washed three times in wash buffer I (mM: 150 NaCl, 2 CaCl₂.2H₂O, 0.8 NaH₂PO₄.2H₂O, 1 MgCl₂, 5 glucose, 25 HEPES; pH 7.4). 500 μl of rubidium buffer (mM: 5.4 RbCl, 150 NaCl, 2 CaCl₂.2H₂O, 0.8 NaH₂PO₄.2H₂O, 1 MgCl₂, 5 glucose, 25 HEPES; pH 7.4) was added for 4 h at 37 °C under an atmosphere of 2 % CO₂. Drugs were added for 30 min after the 4 h incubation period under the same conditions. The rubidium buffer was then removed and the cells were washed three times with 500 μl wash buffer II (mM: 5.4 KCl, 150 NaCl, 2 CaCl₂.2H₂O, 0.8 NaH₂PO₄.2H₂O, 1 MgCl₂, 5 glucose, 25 HEPES; pH 7.4) to remove any remaining rubidium that was not taken up by the cells. To activate HERG, a high K⁺ buffer was added to depolarize the cells. 500 μl of activation buffer (mM: 54 KCl, 150 NaCl, 2 CaCl₂.2H₂O, 0.8 NaH₂PO₄.2H₂O, 1 MgCl₂, 5 glucose, 25 HEPES; pH 7.4) was added for 10 min and then the supernatant was collected into microfuge tubes. To collect the intracellular content of cells, 500 μl of lysis buffer (mM: 150 NaCl, 2 CaCl₂.2H₂O, 0.8 NaH₂PO₄.2H₂O, 1 MgCl₂, 5 glucose, 25
HEPES and 1% Triton-X 100; pH 7.4) was added for 10 min and the solution was collected. The lysis buffer contained Triton-X 100, a detergent which breaks down the cells membrane; leading to the release of intracellular contents. If the samples were not immediately analysed for rubidium content, they were stored at 4 °C.
2.3.6.1.3 **Rb⁺ determination using atomic absorption spectroscopy**

Rubidium content of samples was analysed by flame atomic absorption spectroscopy. The apparatus used was a Varian 220 spectrometer. The samples were diluted (10-fold) with ionisation buffer (2000 μg/ml KCl in 1 % HNO₃ v/v) to suppress ionisation losses of rubidium during atomisation. Samples were sprayed into the flame (acetylene gas burner) via a spray chamber attached to an aspirator. The measured wavelength was 780 nm in the presence of an appropriate hollow-cathode lamp. Rubidium content was calculated by comparison with rubidium standard curves obtained using RbCl (Sigma). The standards were prepared in ionisation buffer in a range between 0 – 5 mg/L (Figure 2-5). The detection limit of rubidium was in the μg/L levels. Efflux percentage was determined by dividing the extracellular content by the total rubidium content (intracellular and extracellular). Absorbance values fell within a range of 0.01-0.4 A₇₈₀.

Rubidium Efflux (%) = [Rb⁺] in supernatant (500μl)/ ([Rb⁺] in lysate (500 μl) + [Rb⁺] in supernatant (500 μl)).

Normalized Rb⁺ efflux was calculated by dividing efflux at each test drug concentration from each well by the maximum efflux obtained in the absence of the test drug.
Figure 2-5: Rb⁺ standard curve.

The standard curve was plotted to determine the concentration of Rb⁺ in samples. Absorbance readings fell within a range of 0.1 - 0.4 A₇₈₀.
2.3.7 Statistics and calculations

All data were expressed as the mean and standard error of the mean (SEM) with the number of observations (n) being given. Data manipulation, IC\textsubscript{50} calculations and graphical presentations were completed in GraphPad Prism 4 software package (GraphPad software Inc, San Diego, USA).

The IC\textsubscript{50} value of drugs is defined as the concentration required to reduce the K\textsuperscript+-induced Rb\textsuperscript+ efflux to 50% of the maximum efflux. For this calculation background efflux in the absence of K\textsuperscript+ was subtracted (typically ~7%).

Dose-response curves were fitted by a standard Hill equation to calculate the IC\textsubscript{50} value as follows:

\[
\text{Fractional block} = \frac{1}{1 + \left(\frac{\text{IC}_{50}}{[\text{drug}]}\right)^n}\]

Where IC\textsubscript{50} is the concentration at which half the maximal inhibition of HERG currents occurs and \(n\) is the Hill coefficient for the fit. The fit is a non-linear sigmoidal dose-response (variable slope). The fit assumes that at maximal inhibition occurs at 1.0 and minimal occurs at 0.0 on the y-axis. Hence, the IC\textsubscript{50} falls at 50% of inhibition which occurs at 0.5.

For statistical analysis between two curves ANOVA was used followed by Bonferroni test if comparing between the same membrane potential on each curve.

For statistical analysis between two points Student’s t-test was used.
For statistical analysis between a point and several points on the same curve, ANOVA was used followed by Dunnett’s test.

*Patch clamp:* Activation curves were fitted using a Boltzmann equation to determine the half maximum activation voltage as follows:

\[
I = \frac{I_{\text{max}}}{1 + \exp \left(\frac{(V_{1/2} - V_m)}{k}\right)}
\]

Where \(I\) is the tail HERG current amplitude at the test potential \(V_m\), \(I_{\text{max}}\) is the maximal HERG tail current observed, \(V_{1/2}\) is the potential at which HERG current is half maximally activated, \(k\) is the slope factor describing HERG current activation.

### 2.3.8 Patch clamp software configuration

The pClamp software was used for setting up experimental protocols for controlling applied voltages. The voltage clamp protocols used are provided as diagrams in relevant sections of results ([Figure 2-12](#) and [Figure 2-15](#)). These protocols were used as previously described (Zhou, 1998a).

### 2.3.9 Materials

#### 2.3.9.1 Drugs

The drugs used were obtained from various sources as follows: astemizole, terfenadine, pimozide, spironolactone, desipramine HCl, apamin, charybdotoxin, diltiazem HCl, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), atropine purchased from Sigma Aldrich (St Louis, USA).
2.3.9.2 Chemicals

The chemicals used were obtained from various sources as follows: Sodium Chloride (NaCl), D-(+)-Glucose, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), ethylene glycol-bis [β-aminoethyl ether]-N, N, N’N’-tetracetic acid (EGTA), rubidium chloride (RbCl), purchased from Sigma Aldrich (St Louis, USA). Magnesium Chloride (MgCl₂.6H₂O), sodium dihydrogen orthophosphate (NaH₂PO₄.2H₂O); purchased from Ajax chemicals. Calcium Chloride dihydrate (CaCl₂.2H₂O) purchased from Merck Pty Ltd (Darmstadt, Germany). Cadmium Chloride (CdCl₂.2.5H₂O) purchased from May & Baker Ltd (Dagenham, England). Agarose-LE-Analytical grade was purchased from Promega (Madison, USA). Tryptone, Bacto-agar and Yeast extract were purchased from Difco laboratory (Becton, Dickinson and Company, Franklin Lakes, USA). Nitric acid (70% w/w) purchased from Ajax Chemicals (Seven Hills, Australia).

2.3.9.3 Plasmid transformation reagents

Luria Broth agar in g: 5 tryptone, 2.5 yeast extract, 5 NaCl, 7.5 agar and 0.2 ml 2 M NaOH, 500 ml H₂O, autoclave.

Luria-Broth medium in g: 5 Tryptone, 2.5 yeast extract, 5 NaCl, and 0.2 ml 2 M NaOH, 500 ml H₂O autoclaved).
SOC medium (in g: 10 Tryptone, 2.5 yeast extract, 0.292 NaCl, 0.093 KCl, 500 ml H₂O autoclaved and pH adjusted to 7.0, 1 ml of 2 M MgCl₂ and 10 ml of 20 % glucose added).

For plasmid digest: Buffer D (mM): 60 Tris HCl (pH 7.9), 1500 NaCl, 60 MgCl₂ and 10 DTT. Phosphate buffered saline (mM): 1.06 Potassium Phosphate monobasic (KH₂PO₄), 155.17 Sodium Chloride (NaCl), 2.97 Sodium Phosphate dibasic (Na₂HPO₄·7H₂O), pH 7.4. Trypan blue (v/v): 0.4 % trypan blue, 0.81 % sodium chloride, 0.06 % potassium phosphate dibasic.

Solutions for midi prep: equilibration buffer (mM: 600NaCl, 100 sodium acetate, pH 5.0, 0.15 % Triton X-100 (v/v)). Suspension buffer (mM: 50 Tris-HCl at pH 8.0, 10 EDTA). Lysis solution (mM: 200 NaOH, 1% SDS (w/v)). Neutralization buffer (3.1 M potassium acetate, pH 5.5). Wash buffer (mM: 800 NaCl, 100 sodium acetate, pH 5.0). Elution buffer (1.25 M NaCl, 100 mM Tris-HCl, pH 8.5).

2.3.9.4 Cell culture reagents

Poly-D-lysine and human serum (from clotted human male whole blood, Type AB, sterile-filtered, (mycoplasma tested, virus tested) purchased from Sigma Aldrich, St Louis, USA. TrypLE Select, Dulbecco’s minimum essential medium (DMEM), Lipofectamine 2000 CD; purchased from Invitrogen Corporation (Carlsbad, USA). Human extracellular matrix and Poly-D-lysine coated 24-well plates purchased from Becton Dickson and Company Biosciences. EX-CELL™ 293 purchased from JRH
Biosciences (Melbourne, Australia). Nunc cell culture ware was purchased from In Vitro Technologies (Melbourne, Australia).

2.3.9.5 PCR reagents

Qiagen OneStep PCR buffer: 20 mM Tris.Cl, 100 mM KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, 1 mM dithiothreitol (DTT); pH 8.7 (20°C). dNTP Mix: 10 mM of each of dATP, dCTP, dGTP, and dTTP; ultrapure quality. Q-Solution: 5x concentrate (no ingredients provided).

2.3.9.6 Patch clamp solutions

External solution contained (mM): 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES; pH 7.4 with NaOH. The osmolarity of the external solution was approximately > 10 % higher than the osmolarity of the internal solution. This was checked by using an osmometer. The osmolarity of the solutions used in this study was adjusted by the addition of sucrose to the external solution.

The electrode internal solution contained (mM): 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES; pH 7.2 with KOH.

2.3.10 Drug preparation

Astemizole, terfenadine, pimozide, haloperidol, and spironolactone were prepared in DMSO (100 %). Quinidine anhydrous and quinidine sulphate were prepared in ethanol (100 %). Desipramine HCl, amitriptyline, diltiazem HCl, clomipramine HCl,
tetraethylammonium chloride (TEA), barium chloride, 4-aminopyridine (4-AP),
atropine, cadmium chloride, apamin, charybotoxin were all prepared in water. All
drugs were prepared at a final stock concentration of 0.01 M and were further diluted
in test buffer at < 0.06 % DMSO; except for TEA and Cd\(^{2+}\) which were prepared at 1
M in H\(_2\)O. Drug aliquots were stored at -20 °C and thawed on the day of
experiments.
2.4 Results

2.4.1 Plasmid sequence

The sequence of the prepared plasmid containing HERG was confirmed by sequencing (Figure 2-6). The resulting sequence was identical to the HERG sequence (accession number U04270). This was confirmed by comparison in nucleotide-nucleotide on BLAST (Figure 2-7). The plasmid was linearized prior to transfection using \textit{Pvu} I (Figure 2-8), an enzyme that cuts the vector at the penicillin gene not required for transfection. Linearizing decreases the likelihood of the vector integrating into the genome in the wrong direction which could lead to a disruption in the expression of the gene during the transfection process. The linearization of the plasmid was confirmed by the presence of a single band when electrophoresed on an agarose gel (0.7 \% w/v). Compared to the original plasmid, which display three bands representing the three forms of DNA known as nicked, linear and supercoiled, the digested plasmid only displayed the linear indicating that the digestion process was successful (Figure 2-8).
Figure 2-6: HERG sequence (Micromon services).

The resulting sequences matched HERG sequence (accession number U04270).
Figure 2-7: Results from the nucleotide-nucleotide search on BLAST for the HERG1 sequence.

The results indicate an identity percentage of 100%. The query entered is the HERG1 sequence obtained from Micromon sequencing facility. The subject is the closest match obtained from the BLAST search, the human putative potassium channel mRNA.
Figure 2-8: HERG plasmid ran on agarose gel

Cut and uncut HERG plasmid ran on a 0.7 % agarose gel. Lane 1 contains λ DNA/EcoR I + Hind III Markers (Promega), lane 2 contains uncut HERG plasmid, and Lane 3 contains cut HERG plasmid with Pvu I. The uncut sample shows three bands which represent nicked, linear and supercoiled DNA. The cut DNA show only the linear form of DNA.
2.4.2 Culture methods

The HEK293 cells were initially adapted to animal-free reagent for culturing. Cells growing in the chemically defined medium EX-CELL 293 at 37 °C and 5 % CO₂ did not show any signs of attachment to the flask surface or growth in monolayers. Ca\(^{2+}\) (0.08 mM) was added as an attachment facilitator; however, cells only attached for one day in its presence and were then floating in suspension (Figure 2-9). Poly-l-lysine was also added to increase cell attachment; however it did not facilitate attachment to the flask. The cells were healthy and increasing in number; however attachment was required for cells to be transfected and so the method was abandoned.

The second approach was to use the standard growth medium-DMEM containing Geneticin and human serum (10 % v/v) (Figure 2-10). Cells were initially attaching to the flasks for a few days and then detaching in monolayers if medium wasn’t exchanged within 2 days or if the cells were cultured in large volumes of medium such as 15 ml. The cells were passaged at 80 % confluence and no more than 7 ml of medium was added to 75 cm\(^2\) flasks. Since these cells did not attach very well at > 80 % confluence, the culture dishes were coated with human extracellular matrix (purified matrix extract derived from human placenta) during transfection experiments to increase attachment.
Figure 2-9: HEK293 cells cultured using EXCELL-293.

Panels A and B: Native HEK293 cells cultured in EXCELL-293 medium. The figure shows signs of attachment following one day of culture. Panel A: x 20 magnification. Panel B: x 60 magnification. Panels C and D: Native HEK293 cells cultured in EXCELL-293 medium after two days in culture. Cells divide in suspension after two days or more in culture. Panel C: x 20 magnification. Panel D: x 60 magnification.
2.4.3 Transfection

After culturing HERG transfected HEK293 cells in DMEM containing human serum (10 % v/v) and Geneticin, more than 25 colonies were evident. These cells were kept in Geneticin-medium during culturing (Figure 2-10). Colonies were expanded out in larger flasks and grown in Geneticin-containing growth medium for 14 days, to allow for stable generation of the HERG protein (Table 2-2). The control wells produced the expected results. Wells containing Geneticin and non-transfected HEK293 cells led to the death of all HEK293 cells after 7 days of culture presumably because the cells were not Geneticin resistant. Wells that contained HEK293 cells with no Geneticin but the transfection complex, showed no signs of cell death, which indicated that the transfection complex was not toxic to the cells. Wells that contained the native HEK293 in growth medium in the absence of Geneticin, showed no signs of cell death, which was expected as a check for the safety of the growth medium.
Table 2-2: Transfection results in the presence of the selective drug-Geneticin.

<table>
<thead>
<tr>
<th>Cell Condition</th>
<th>Geneticin present</th>
<th>Cell growth?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfected HEK293</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Transfected HEK293</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-transfected HEK293</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Non-transfected HEK293</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 2-10: HEK293 and transfected cells cultured using DMEM and human serum

**Panel A:** Native HEK293 cells cultured in growth medium containing human serum, x60 magnification. **Panel B:** HERG-transfected HEK293 cells cultured in growth medium containing human serum and Geneticin, x 20 magnification. **Panel C:** HERG-transfected HEK293 cells cultured in growth medium containing human serum and Geneticin, x 60 magnifications.
2.4.4 PCR

PCR experiments were performed using specific HERG primers. The HERG-transfected HEK293 cells showed a band at 575 bp (Figure 2-11). This band was absent in the native HEK293 cells. As a positive control for HERG, the plasmid used for transfection was also amplified by PCR; the amplified plasmid DNA had the same size as the transfected DNA. This test confirmed the success of the transfection.
Figure 2-11: HERG expression in HEK293 and transfected cells.

PCR products were amplified as per protocol using specific HERG primers (see 2.3.4). The resulting PCR products were electrophoresed on agarose gel (0.7 % w/v) at 70 V for 60 min. Lane 1, 1 Kb DNA ladder, lane 2, native HEK293 cells, lane 3, HERG-transfected HEK 293 cells, lane 4, HERG plasmid in pcDNA3.1.
2.4.5 HERG currents in HERG transfected HEK293 cells using patch clamp electrophysiology

The characteristics of currents in HERG transfected HEK293 cells were studied using whole-cell recordings. A voltage-clamp protocol was used, where cells were clamped at -80 mV and depolarized to voltages within the range of -60 to +50 mV in 10 mV steps each for 4 s to activate HERG currents (Figure 2-12A). The cells were then clamped to -50 mV for 5 s to record a tail current. A tail current is a measure of maximum channel activation at the applied voltage in the absence of inactivation. A trace of the currents obtained using the protocol is shown in Figure 2-12C and Figure 2-13. The trace exhibits reported properties of HERG currents (Zhou, 1998a) where depolarization steps caused an increase in outward current at voltages positive to -50 mV which reached its maximum at 0 mV. At voltages higher than 0 mV, the currents decreased. This steep negative slope is a property of inward rectification of HERG caused by voltage-dependent inactivation of the channel. The non-transfected native HEK293 cells had minimal membrane current, the current was ohmic and voltage independent (Figure 2-12B and Figure 2-14).

Figure 2-12D represents the current-voltage (I - V) plot of peak currents obtained during each depolarisation step. Current increased in voltage steps from -60 mV to 0 mV and the maximum outward current occurred at 0 mV. At positive membrane potentials, current subsided which indicates inward rectification. The peak tail currents recorded at -50 mV were used to construct an activation curve for normalized HERG currents (Figure 2-12E). The calculated half-maximum activation voltage ($V_{1/2}$) from the activation curve for HERG was -23.0 mV ± 1.5 and the slope
factor was $9.5 \pm 1.3$ at $35 \, ^\circ\text{C}$. These values were obtained by fitting the data with a Boltzmann curve.

The same voltage protocols were also used to measure currents in native HEK293 cells. **Figure 2-12B** is a trace of current recorded from a single native HEK293 cell exhibiting a small outward current that did not show any voltage-dependence. These currents could be due to endogenous background channels expressed in the cells. The currents obtained in native HEK293 cells were presented as an I - V plot along with the fully activated I - V plot of HERG in HERG transfected HEK293 cells in **Figure 2-12D**, a linear but small increase in outward current was observed, independent of voltage.

The current-voltage (I-V) relation for HERG currents during repolarisation to detect voltage dependence of inactivation was examined by applying different voltages as shown in **Figure 2-15** and **Figure 2-16**. The cells were held at -80 mV and depolarised to +60 mV for 1 s. The cells were then repolarised to voltages within the range of -100 mV to +40 mV to measure HERG currents. The decrease in voltage from +60 mV allowed for recovery from inactivation of HERG currents, which displayed a maximum at -20 mV. The mean I - V relationship was plotted (**Figure 2-17**). The mean I-V plot of tail currents showed data typical to that seen in a single cell trace (**Figure 2-15**), where the maximum current reached was between -30 mV to -20 mV and inward rectification was evident at voltages positive to -20 mV (see **Figure 2-17** for mean data).
To confirm that the currents detected were due to HERG activation in these cells, the known HERG inhibitor terfenadine was tested. Terfenadine was added in the external bathing solution for 5 min prior to recording using the activation voltage clamp protocol. Results ([Figure 2-18](#)) show a decrease in the current caused by the step voltages applied. At 10 μM terfenadine, a negative tail current was detected presumably due to toxicity of cells caused by such a high concentration as evidenced by gradual increases in leak currents (not shown).
Fig 2-12

A

+50

mV

-80

-60

-50

4 s

5 s

B

nA

2.0

1.5

1.0

0.5

0.0

1 s

C

nA

2.0

1.5

1.0

0.5

0.0

1 s

D

Current (nA)

-0.2

0.0

0.2

0.4

0.6

0.8

1.0

-80

-60

-40

-20

0

20

40

60

Membrane potential (mV)

E

Normalized tail current

-0.2

0.0

0.2

0.4

0.6

0.8

1.0

-80

-60

-40

-20

0

20

40

60

Membrane potential (mV)
Figure 2-12: Consolidated currents from data in Figure 2-13 and Figure 4-2 and data analysis.

Panel A: Voltage Clamp protocol used for patching HEK293 cells to detect HERG currents. With a holding potential of -80 mV, the cells were depolarised to potentials within the range of -60 mV to 50 mV in sequential 10 mV steps over 4 s and then clamped to -50 mV for 5 s to measure tail currents. Panel B: Outward current measured in native HEK293 cells. Panel C: HERG current measured in transfected HEK293. Voltage clamp protocol outlined in panel C was applied to obtain activation currents. Panel D: The current-voltage (I - V) plot of HERG currents in HERG transfected HEK293 cells (○, n = 4) and the background current in native HEK293 cells (○, n = 4) measured at the end of each depolarization step. Panel E: The activation curve plotted using HERG tail currents in HERG transfected HEK293 cells (●, n = 13) and background currents in native HEK293 cells (○, n = 4). Mean data was fitted with a Boltzmann relationship to obtain a $V_{1/2}$ of -23.0 mV ± 1.5 for HERG activation voltages. * Denotes significantly different from native HEK293 (P< 0.05, ANOVA, followed by Bonferroni test).
Figure 2-13: Typical HERG currents measured in a single HERG transfected HEK293 cells using the protocol outlined in Figure 2-12.

Each individual panel represents current measured at the applied voltage indicated. The consolidated data is shown in Figure 4-2. The time scale is shown at the base of the -60 mV panel.
Figure 2-14: Currents measured using HERG activation protocol measured in native HEK293 cells.

The each individual panel represents current measured at the applied voltage. The consolidated data is shown in Figure 4-2. The time scale is shown at the base of the -60 mV panel.
Figure 2-15: The activated I-V relation of HERG currents in HERG transfected HEK293 cells.

Panel A: HERG currents obtained using the voltage protocol outlined in panel B. The current amplitude for this cell reached a maximum at -20 mV. Panel B: The voltage clamp protocol used to measure HERG currents inactivation. The cells were held at -80 mV and depolarised to -60 mV, voltages within the range of -100 mV to -40 mV were applied to measure voltage dependence of HERG tail currents.
Figure 2-16: Typical HERG tail currents measured in a single HERG transfected HEK293 cells using the protocol outlined in Figure 2-15.

Each individual panel represent tail current measured at the applied voltage indicated. The time scale is shown at the base of the -100 mV panel.
Figure 2-17: Current-Voltage (I - V) plot of peak HERG tail currents measured in HERG transfected HEK293 cells in Figure 2-15 and Figure 2-16.

The plot represents the tail currents detected at each applied membrane potential of the cell. Data represents mean (n = 4) ± SEM.
Figure 2-18: The effect of terfenadine and time on activation curves respectively.

Panel A: The effect of terfenadine on the activation curve of HERG tail currents in HERG transfected HEK293 cells measured room temperature. The currents were obtained using the voltage protocol outlined in Figure 2-12. Drugs were applied for 5 min prior to taking measurements. Increasing concentrations of terfenadine were tested in a single cell. Panel B: A time-control of HERG currents in transfected HEK293 cells in the absence of terfenadine. Recordings were taken every 3 min showing stability of the responses. Data represents n ± SEM, n = 4.
2.4.6 Rubidium assay

2.4.6.1 Basic characteristics of Rb⁺ uptake

The rubidium efflux assay was used to monitor K⁺ channel activity. Experiments were conducted to determine the characteristics of Rb⁺ uptake and release in HERG transfected HEK293 cells and native HEK293 cells. Incubation of cells with RbCl (5.4 mM) for 180 min resulted in Rb⁺ accumulation in HEK293 cells. When the cells were lysed in 500 μl lysis buffer, the concentration of Rb⁺ in the samples was 21 mg/L on average (n = 47) in transfected HEK293 cells and 28 mg/L in native HEK293 cells in a well containing 1 x 10⁵ cells (see Table 2-3). This indicates that both cells accumulated Rb⁺ to a similar extent.

2.4.6.2 Basic characteristics of K⁺ induced Rb⁺ release

The effect of K⁺ depolarization was measured under various conditions to optimize a K⁺ depolarization protocol. K⁺ (5.4 - 60 mM) concentration-dependently increased Rb⁺ efflux when measured at a fixed time point of 10 min (Figure 2-19). The K⁺-induced Rb⁺ efflux was time dependent with the most rapid changes in the first 10 min (Figure 2-20). When cells were depolarized using a buffer containing high K⁺ (54 mM), the percentage Rb⁺ in transfected cells which was released was 60 % at 10 min and this was significantly higher than the native HEK293 cells (14 %) (Figure 2-21). This latter value was comparable to the basal efflux in the non-depolarised transfected cells in the same time period. The HERG K⁺ blocker terfenadine (Roy, 1996) was used to inhibit Rb⁺ release. Terfenadine (0.01 mM) reduced the efflux to baseline levels in HERG transfected HEK293 cells (Figure 2-22). Furthermore, high
K⁺ buffer (54 mM) was tested over 40 min in the presence and absence of a potent HERG blocker-astemizole (Suessbrich, 1996a) (Figure 2-23). Since astemizole inhibits the HERG K⁺ channel function in the nanomolar range (Suessbrich, 1996a), 1 µM was tested. The 1 µM astemizole containing buffer significantly reduced the efflux to values obtained in baseline (Figure 2-20). It is likely that the remaining efflux not blocked by astemizole was due to background channels.
Table 2-3: Cellular Rb+ uptake

Comparison of Rb+ uptake in HERG transfected HEK 293 cells and native HEK293 cells. Intracellular measurements were taken by lysing cells using a buffer containing Triton X-100. Samples were collected 10 min after incubation with lysis buffer. Samples were diluted and Rb+ content was analysed using atomic absorption spectroscopy.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Rb+ uptake (mg/L)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HERG Transfected HEK293</td>
<td>21.0 ± 0.6</td>
<td>47</td>
</tr>
<tr>
<td>Non-transfected HEK293</td>
<td>28.1 ± 1.1</td>
<td>12</td>
</tr>
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</table>
Figure 2-19: Effect of different $K^+$ concentrations on $Rb^+$ efflux in HERG-transfected HEK293 cells.

The contact time with $K^+$ was 10 min. The percentage of $Rb^+$ efflux in the supernatant to the total $Rb^+$ content (supernatant and inside cell) was taken as a measure of HERG function in the HERG transfected cells. Data represents mean at each KCl concentration ($n = 2$).
Figure 2-20: Effect of contact time of [K+] on Rb+ efflux in HERG-transfected HEK293 cells.

Data represents mean efflux from 3 wells ± SEM. The percentage of Rb+ efflux in the supernatant to the total Rb+ content (supernatant and inside cells) was taken as a measure of HERG function in the HERG transfected HEK293 cells. * Denotes significantly different from 5.4 mM (P < 0.05, ANOVA).
Figure 2-21: Comparison of the effect of K⁺-induced Rb⁺ efflux in native HEK293 and HERG transfected HEK293 cells.

The Rb⁺ efflux in the supernatant as a percentage of the total Rb⁺ content (supernatant and inside cells) under basal conditions over 10 min and in the presence of KCl (54 mM) was measured. Data represents mean ± SEM (n = 6). * Denotes significantly different from basal (P < 0.05, Student’s t-test).
Figure 2-22: Comparison of the effect of terfenadine on K⁺-induced Rb⁺ efflux in native HEK293 and HERG transfected HEK293 cells.

Rb⁺ was induced by high KCl solution (54 mM) for 10 min. The percentage of Rb⁺ efflux in the supernatant to the total Rb⁺ content (supernatant and inside cells) was measured (Rb⁺ efflux %). The K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells was inhibited by various concentrations of terfenadine. Data represents mean ± SEM (n = 6). * Denotes significantly different from basal (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-23: Effect of astemizole on K⁺ evoked Rb⁺ efflux in HERG-transfected HEK293 cells.

The percentage of K⁺-induced Rb⁺ efflux in the supernatant to the total Rb⁺ content (supernatant and inside cells) was calculated at various time points after the addition of K⁺ in the absence (control) and presence of astemizole (1 μM). Data represent mean efflux from 3 wells ± SEM (n = 3). * Denotes significantly different from control (P < 0.05, ANOVA).
2.4.6.3 Effect of non-HERG K\(^+\) channel blockers on Rb\(^+\) release

The effect of K\(^+\) channel blockers was tested in the system to determine whether other K\(^+\) channel inhibitors affect Rb\(^+\) efflux. Data obtained in these experiments (see sections below) did not show any inhibition of the K\(^+\) induced Rb\(^+\) efflux by inhibitors such as tetraethylammonium, barium and atropine. However, 4-aminopyridine showed a concentration-independent block of Rb\(^+\) efflux.

2.4.6.3.1 Tetraethylammonium

Tetraethylammonium, an inhibitor of Ca\(^{2+}\) activated K\(^+\) channels (Kaczorowski, 1999), voltage-gated K\(^+\) channels such as KNCQ channels (see section 1.1.2.3) was tested to determine its effect on K\(^+\)-induced Rb\(^+\) efflux in HERG transfected HEK293 cells. Tetraethylammonium (1 nM - 10 mM) had a very small facilitatory effect on K\(^+\) (50 mM) evoked Rb\(^+\) efflux in these cells but this was not concentration dependent (Figure 2-24). All data were normalized to the efflux in the absence of the drug.

2.4.6.3.2 Ba\(^{2+}\)

Ba\(^{2+}\) an inhibitor of many ion channels including the delayed rectifier K\(^+\) channels (Taglialatela, 1993b) was tested to determine its effect on K\(^+\)-induced Rb\(^+\) efflux in HERG transfected HEK293 cells. Ba\(^{2+}\) (1 nM - 30 µM) had a small facilitatory effect on K\(^+\) (50 mM) evoked Rb\(^+\) efflux in these cells but this was not concentration dependent (Figure 2-25). All data were normalized to the efflux in the absence of the drug.
2.4.6.3.3 Atropine

Atropine, a muscarinic receptor antagonist required to inhibit M activated K+ currents was tested to determine its effect on K+ induced Rb+ efflux in HERG transfected HEK293 cells. Atropine (1 nM – 30 μM) had a facilitatory effect on K+ (50 mM) evoked Rb+ efflux in these cells (Figure 2-26). All data were normalized to the efflux in the absence of the drug.

2.4.6.3.4 4-aminopyridine

4-aminopyridine (4-AP), an inhibitor of voltage gated K+ channels (see section 1.1.2.3) was tested to determine its effect on K+ induced Rb+ efflux in HERG transfected HEK293 cells (Figure 2-27). 4-AP (1 nM – 30 μM) had a small inhibitory effect on K+ (50 mM) evoked Rb+ efflux in these cells but the effect was not concentration dependent (Figure 2-27). All data were normalized to the efflux in the absence of the drug.
Figure 2-24: Tetraethylammonium concentration-curve

Effect of tetraethylammonium (TEA) on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 16 TEA concentrations were used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 10 mM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-25: Ba²⁺ concentration-curve

Effect of barium (BaCl₂) on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 Ba²⁺ concentrations were used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
**Figure 2-26: Atropine concentration-curve**

Effect of atropine on K+-induced Rb+ efflux in HERG-transfected HEK293 cells. Rb+ efflux over 10 min period in each well was expressed as a ratio of total Rb+ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K+-evoked Rb+ efflux in the presence of 10 atropine concentrations were used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-27: 4-Aminopyridine concentration-curve

Effect of 4-aminopyridine (4-AP) on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 4-AP concentrations was used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes curve was significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test). Individual points were not significant from 1.
2.4.6.4 Effect of HERG K⁺ channel blockers on Rb⁺ release

Several structurally different drugs known to inhibit HERG were tested using the Rb⁺ efflux assay. The effect of these drugs on the Rb⁺ efflux was tested to determine whether the effect is concentration-dependent. All data collected were normalized by dividing efflux values at different concentration point by the maximum efflux obtained in the absence of tested drugs.

2.4.6.4.1 Pimozide

Pimozide (1 nM – 30 μM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-28). The maximum inhibition of Rb⁺ efflux at 30 μM was 92 % (Figure 2-28). The IC₅₀ value for pimozide was 38.70 ± 0.06 nM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.99.

2.4.6.4.2 Haloperidol

Haloperidol (1 nM – 30 μM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-29). The maximum inhibition of Rb⁺ efflux at 30 μM was 84 % (Figure 2-29). The IC₅₀ value for haloperidol was 376.00 ± 0.04 nM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.99.
2.4.6.4.3 Amitriptyline

Amitriptyline (1 nM – 30 μM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-30). The maximum inhibition of Rb⁺ efflux at 30 μM was 36 % (Figure 2-30). The IC₅₀ value obtained from the curve was 13708.0 ± 0.2 nM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.96.

2.4.6.4.4 Quinidine anhydrous

Quinidine anhydrous (1 nM – 30 μM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-31). The maximum inhibition of Rb⁺ efflux at 30 μM was 65 % (Figure 2-31). The IC₅₀ value obtained from the curve was 7.45 ± 0.14 μM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.97.

2.4.6.4.5 Quinidine sulfate

Quinidine sulfate (1 nM – 30 μM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-32). The maximum inhibition of Rb⁺ efflux at 30 μM was 71 % (Figure 2-32). The IC₅₀ value obtained from the curve was 3.65 ± 0.05 μM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.99.
2.4.6.4.6  Astemizole

Astemizole (1 nM – 30 µM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-33). The maximum inhibition of Rb⁺ efflux at 30 µM was 84 % (Figure 2-33). The IC₅₀ value obtained from the curve was 62.00 ± 0.03 nM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.99.

2.4.6.4.7  Terfenadine

Terfenadine (1 nM – 30 µM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-34). The maximum inhibition of Rb⁺ efflux at 30 µM was 82 % (Figure 2-34). The IC₅₀ value obtained from the curve was 260.00 ± 0.06 nM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.98.

2.4.6.4.8  Cadmium

Cadmium Cl₂ (10 nM - 300 µM) concentration-dependently inhibited K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-35). The maximum inhibition of Rb⁺ efflux at 30 µM was 74 % (Figure 2-35). IC₅₀ value obtained from the curve was 60.9 ± 0.026 µM (Table 2-4) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.99.
2.4.6.4.9 Desipramine

Desipramine (1 nM – 30 µM) inhibited K⁺-evoked Rb⁺ efflux HERG transfected HEK293 cells (Figure 2-36). The maximum inhibition of Rb⁺ efflux at 30 µM, the maximum concentration tested was 31 % (Figure 2-36). No IC₅₀ value could be obtained from the curve and so was referred to as > 30 µM (Table 2-4). The plotted curve had a fit coefficient (r²) of 0.67.

2.4.6.4.10 Spironolactone

Spironolactone (1 nM – 30 µM) had a small inhibitory effect on K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-37). The maximum inhibition of Rb⁺ efflux at 30 µM, the maximum concentration tested was 3 % (Figure 2-37). No IC₅₀ value could be obtained from the curve and so was referred to as > 30 µM (Table 2-4).

2.4.6.4.11 Diltiazem

Diltiazem (1 nM – 30 µM) did not inhibit K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells (Figure 2-38). The maximum inhibition of Rb⁺ efflux at 30 µM, the maximum concentration tested was 5 % (Figure 2-38). No IC₅₀ value could be obtained from the curve and so was referred to as > 30 µM (Table 2-4).
Figure 2-28: Pimozide concentration-curve

Effect of pimozide on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 pimozide concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 38.70 ± 0.06 nM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9915. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-29: Haloperidol concentration-curve

Effect of haloperidol on K+-induced Rb+ efflux in HERG-transfected HEK293 cells. Rb+ efflux over a 10 min period in each well was expressed as a ratio of total Rb+ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K+-evoked Rb+ efflux in the presence of 10 haloperidol concentrations was used to construct a concentration-response curve. The IC$_{50}$ value obtained from the curve was 376 ± 0.037 nM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient ($r^2$) of 0.9978. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-30: Amitriptyline concentration-curve

Effect of amitriptyline on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 amitriptyline concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 13708 ± 0.15 nM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9636. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-31: Quinidine anhydrous concentration-curve

Effect of quinidine anhydrous on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 quinidine anhydrous concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 7.45 ± 0.14 μM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9705. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-32: Quinidine sulfate concentration-curve

Effect of quinidine sulfate on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 quinidine sulfate concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 3.65 ± 0.05 μM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9957. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-33: Astemizole concentration-curve

Effect of astemizole on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 astemizole concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 62 ± 0.03 nM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9931. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-34: Terfenadine concentration-curve

Effect of terfenadine on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 terfenadine concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 260 ± 0.06 nM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9836. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-35: Cadmium Cl₂ concentration-curve

Effect of cadmium Cl₂ on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 cadmium concentrations was used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 60.9 ± 0.026 μM and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9922. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-36: Desipramine concentration-curve

Effect of desipramine on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 desipramine concentrations was used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 µM. The data was fitted using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.67. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 2-37: Spironolactone concentration-curve

Effect of spironolactone on K+-induced Rb+ efflux in HERG-transfected HEK293 cells. Rb+ efflux over a 10 min period in each well was expressed as a ratio of total Rb+ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K+-evoked Rb+ efflux in the presence of 10 spironolactone concentrations was used to construct a concentration-response curve. No IC$_{50}$ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
**Figure 2-38: Diltiazem concentration-curve**

Effect of diltiazem on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of the drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 diltiazem concentrations was used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
2.4.6.5 Comparison of Rb+ efflux and patch clamp studies

Electrically stimulated HEK293 cells transfected with HERG generate a current carried by the outward flow of K+. This can be measured by patch clamp electrophysiology. In this section literature values for inhibition of HERG by drugs using the patch clamp technique were compared to those obtained using the K+-evoked Rb+ efflux in this study (Table 2-4). There was a strong relation for the drugs between both techniques when fitted with best line of fit with a correlation coefficient ($r^2$) of 0.76.
Correlation between IC$_{50}$ values obtained using patch clamped HERG-HEK293 cells obtained from the literature and Rb$^+$ assay using HERG transfected HEK293 cells. The data was fitted with a line of identity shown as a dashed line. The regression line is shown as a solid line. The regression line, $y = 0.72x + 1.38$ resulted in a correlation coefficient ($r^2$) of 0.76 for the 7 positive drugs tested.

Figure 2-39: Correlation of IC$_{50}$ values from Rb$^+$ assay and patch clamp
Table 2-4: Comparison of IC\textsubscript{50} values obtained using the Rb\textsuperscript+ assay in HERG transfected HEK293 cells and literature.

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<th>Literature</th>
<th>Current Study</th>
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<td>Patch Clamp IC\textsubscript{50} (nM)</td>
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2.5 Discussion:

2.5.1 Choice of cell line

Inhibition of the HERG K+ channel by various drugs has lead to the need for the development of preclinical in vitro assays to determine potential inhibition of the channel by new candidate drugs (see Introduction). To date, in vitro assays have involved the use of heterologous cell lines (transfected) for high throughput testing. These heterologous cell lines are favoured over constitutive (native) cell lines for several reasons including (1) the density of transfected genes is at least ~50 fold higher than that constitutive under normal culture conditions which decrease signal to noise levels (Taglialatela, 1998), (2) heterologous cell lines such as HEK293 and CHO do not exhibit any signalling characteristics that are present in the cardiovascular system (Cavero, 2000; Witchel, 2002; Zhou, 1998a; Zhu, 1998), eliminating any extraneous factors that may contribute to the response, and (3) stable transfection of these cells generates stable expression of the inserted genes over a long period. Generally, mammalian cell lines are also favoured over amphibian systems for testing of drug candidates. Testing in mammalian cells provides the flexibility for temperature control allowing recordings at physiological temperatures (Zhou, 1998a). The use of mammalian cell lines, specifically human cell lines may provide a better presentation of human response. Testing in Xenopus oocytes produces false estimations of drug IC50 values compared to mammalian systems (Taglialatela, 2000; Weerapura, 2002). Taglialatela et al (2000) has shown that there is a 10-fold difference in the IC50 values obtained for mizolastine blockade of the HERG K+ channel in Xenopus oocytes and in HEK293, which the author explains is due to the fact that mammalian membrane properties differ to that of amphibians which
might impede the access of compounds. Taglialetela et al (1998) has also shown the same issue with loratidine in Xenopus oocytes. This could be due to the presence of the large yolk in the oocyte in which lipophilic material interacts with test substances resulting in underestimated potency values (Witchel, 2002). These results indicate that amphibian systems are not ideal for drug safety testing as these systems produce underestimated data on drug inhibition. It is also unsuitable for high throughput screening. Hence, the HEK293 cell line was chosen for the present study as it provides a better presentation of human and shows more consistency in its stable expression compared to other mammalian cell lines such the CHO cell line which quickly lose their expression level with culture, see review (Witchel, 2002).

### 2.5.2 Culture using animal-free reagents

Cell culture was first developed in 1885 by isolating a chick embryo and culturing it in vitro using its humoral fluids, see review by (Jayme, 1985). The required components for culturing cells in vitro were then defined and led to the development of the first chemically defined growth medium known as medium 199 for the culture of chick embryo cells (Morgan, 1950). It was later discovered that cells such as L cell mouse fibroblasts and HeLa cells required the addition of serum (Eagle, 1955a; Eagle, 1955b; Eagle, 1957; Fisher, 1958), which then became an important component of successful cell culturing. Generally, growth mediums contain components required for cell proliferation and division such as vitamins, amino acids, inorganic salts and other components such as glucose (Eagle, 1955b; Griffiths, 1987; Jayme, 1985). Serum is essential in cell culture as it contains several components required for the proliferation of mammalian cell lines including growth factors such as platelet-
derived growth factors (Stiles, 1983), hormones, nutrients and attachment factors (Griffiths, 1987; Shah, 1999). Hormones are essential for cell growth and function, growth factors are required as mitogens and for cell function and proteins such as fibronectin are required for attachment and spreading factors (Shah, 1999). The most commonly used sera in culture are isolated from foetal bovine, calf or horse depending on the cell line cultured. This study reports the growth of human embryonic kidney (HEK293) cells using human serum and the defined medium DMEM. Cells quickly adapted to the growth conditions compared to EXCELL293 medium and no change in cell morphology was seen. Human serum is rarely used in cell culture but has been proposed for fastidious human cell line, see review by (Shah, 1999).

There are arguments for routine use of human serum as opposed to animal serum. Animal sera have been shown to have viral contamination in various batches which if not detected would lead to the infection of cultured cell lines (Erickson, 1991; Nuttall, 1977). Animal sera are also a potential source of microbial infections. All batches of animal sera must be tested for viral contamination prior to packaging, 20 to 50 % of commercial foetal bovine serum is positive to viral contamination (Wessman, 1999). For human serum, only healthy individuals with negative results to viral infections such as HIV and Hep are allowed to donate blood which is performed before any donor blood is collected. Human sera should also provide a healthier environment for mammalian cell cultures such as those derived from human as it provides growth in their natural environment. Human sera uses in cell culture have been reported for dendritic cells and human embryonic stem cells
(Ellerstrom, 2006; Royer, 2006). The benefits of using human sera are several as it contains high levels of epidermal derived growth factors and platelet derived growth factors (Ayache, 2006; Tallheden, 2005). These factors play an important role in the regulation of cell growth, proliferation and differentiation. This present study provides evidence that human sera can be used successfully in the growth of immortalized human cell lines. An additional factor that was used in the present study is human extracellular matrix derived from human placenta. The human extracellular matrix was used during transfection to minimise cell detachment which could occur due to the decrease in serum supplements.

Chemically defined mediums which do not require serum for cell growth have been developed for several cell lines, including the HEK293 cells, CHO cells and HeLa cells (SAFC-Biosciences, 2006a; SAFC-Biosciences, 2006b; SAFC-Biosciences, 2006c). These mediums support cell culture in suspension and do not support adherent cells. The current study required adherent cells for the development of the rubidium assay and for the patch clamp technique. Adherent cells make it easier to select transfected clones during the selection process with Geneticin as only successful transfected cells remain adhered to culture flasks compared to non-transfected cells which undergo apoptosis due to exposure to the selection drug. In the current study, alternate methods were also tested and the chemically defined medium EX-CELL293 was used to culture HEK293 cells (Ferreira, 2005; SAFC-Biosciences, 2006b). To modify the medium, calcium was added and this supports cell adherence by facilitating interactions between proteins on the plasma membranes of cells (Kvansakul, 2004; Liao, 2006; Takeichi, 1977). However, cell adherence was only evident for a short
period in culture which indicates the necessity for the growth factors found in serum for cell adherence for longer periods and the method was not used further.

Cell lines growing in attached monolayers require dissociation from cultured flasks prior to passaging. The most commonly used dissociation substance is trypsin which is obtained from pigs. This substance was replaced in this study by an animal-free dissociation enzyme known as TrypLE Select which was derived from a bacterial source (GIBCO-Invitrogen, 2004b). TrypLE Select has similar kinetic and cleavage properties to trypsin, however, unlike trypsin it shows significant stability at 4 °C and 37 °C and can be employed in serum-free and serum-supplemented systems (GIBCO-Invitrogen, 2004c). The use of TrypLE Select decreases the chances of viral contamination of cultured cells caused by viruses carried in pigs (the source of trypsin) (Riteau, 2006). Serum contains proteins that inhibit protease activity of trypsin and is used experimentally to inhibit its action. TrypLE Select does not require serum (observed in this study) and its actions were inhibited simply by washing the cells in serum-free medium. Thus, TrypLE select use decreases the need for serum. TrypLE Select use was therefore more efficient than trypsin. Indeed, a volume of 1 ml per 75 cm² culture flask was efficient for cell detachment over a 2 min period at room temperature, this is consistent with previously reported in this cell line (GIBCO-Invitrogen, 2004c). In summary, TrypLE Select provides a non-animal dissociation system with advantages over animal derived trypsin.
2.5.3 Transfection of HEK293 cells

An important aspect of this work is the establishment of a stably transfected cell line with the HERG gene using animal-free methodology. Transfection of the cells using reagents derived from animal-free origin was successful. This is one of the first reports of eliminating the use of animal-derived products in inserting genes into cells and the first for HERG. There are several methods for transfection of genes including electroporation, the calcium phosphate method and the use of lipophilic reagents, see review by (Colosimo, 2000; Witchel, 2002). The reagent used for transfection in this study was the lipofectamine CD 2000, an animal-origin free lipophilic lipid detergent that forms a complex with DNA that enters cell membrane. This reagent has the same effect as the animal-derived form known as lipofectamine except that its components were derived from non-animal origin. Studies have shown the high efficiency of transfection using lipofectamine with little cell damage (Rocha, 2002) compared to other methods such as electroporation which causes cell death, see review by (Gehl, 2003). Lipofectamine CD 2000 has shown similar transfection results as standard lipofectamine 2000 (derived from animal-origins) when tested in several cell lines including COS-7L, CHO, HEK293 and BHK-21 (Invitrogen, 2005).

Stable transfection was chosen over transient as it is more convenient to use a cell line that expresses the HERG protein at a constant level. Transient transfection allows for the expression of the gene for a period of 72 hours, however stable transfection allows for a constant expression of the protein over several months in culture (Colosimo, 2000). Stable transfection occurs where the DNA construct is integrated into the cell genome and thus is processed as part of the cells function at a constant
level (Pagliaro, 2001). This was achieved in the present study by treating transfected cells with a selection drug (Geneticin) which only allows transfected cells to proliferate as these express a gene encoding resistance to the drug in the plasmid vector. Stable transfection is considered a valuable tool in high throughput screening (Pagliaro, 2001) and thus was chosen for the development of the Rb⁺ assay in HEK293 cells. The HERG gene has been previously stably transfected in HEK293 cells using lipofectamine and other animal-derived products (Zhou, 1998a). The results of Zhou’s study were similar to those obtained using transient expression (Snyders, 1996b) and similar to this present study using animal-free methods (see section 2.5.5).

2.5.4 Molecular data

Using primers specific for HERG DNA (Arcangeli, 1998), a single band was detected in HERG transfected HEK293 cells. The band detected was of similar size to that in the amplified plasmid used for transfection and previously reported (Arcangeli, 1998; Shoeb, 2003). PCR was used as it provides a useful tool for RNA detection using animal-free reagents. Western blotting is commonly used to detect the success of the transfected gene by measuring the protein level, see reviews by (Kurien, 2006; Pagliaro, 2001). This method was not employed in the study as it requires the use of antibodies raised in animals to detect a binding site on the protein, see review by (Harper, 1990). However, since western blotting was not used to determine the expression level of the protein, patch clamp was used as indicator of HERG protein expression by detecting HERG function. In patch clamp, the channel activation
parameters were similar to those previously reported (Zhou, 1998a) (see section 2.5.5).

2.5.5 HERG characteristics in HERG transfected HEK293 using patch clamp electrophysiology

In the current study, the results of transfection using animal-free methods were explored by examining the characteristics of HERG K⁺ channels in HERG transfected HEK293. The currents obtained in this study had properties comparable to those obtained previously in this cell line at 35°C (Zhou, 1998) and this is described below.

In the present study, depolarization caused an increase in outward current at voltages positive to -40 mV but was decreased at voltages positive to 0 mV similar to Zhou (1998a). This decrease in outward current is caused by inactivation which occurs due to an increase in the proportion of channels turning to the inactive state. In the present study, inactivation of the channel was clearly observed at positive voltages which causes inward rectification as previously reported (Trudeau, 1995). Measuring tail currents allowed demonstration of the transfer of the channels from the inactive to the active and then the deactivated state. In the present study, tail currents displayed a rapid increase in the outward current followed by a slow deactivation rate at -50 mV, which is a characteristic of HERG channels in other studies (Vandenberg, 2000). The activation curve constructed from the tail currents showed that a maximum outward current in the cell line was reached at voltages positive to 0 mV and this agrees with previous reports in oocytes and transient transfected HEK293 cells (Sanguinetti, 1995; Trudeau, 1995).
To further understand the mechanisms by which the HERG K+ channels change from one state to another, voltage dependence of channel inactivation during repolarization was employed. The results of the repolarization protocol further confirmed that HERG passes maximum current at repolarization voltages between -20 and -30 mV, which supports its role in the repolarization phase of the ventricular action potential (Zhou, 1998a).

The results of the study provide evidence that stable transfection using non-animal methods has been achieved and has resulted in the production of fully functional HERG K+ channels. The patch clamp studies were performed over several months after cell transfection and HERG K+ currents were still observed.

2.5.6 Rubidium efflux assay

Rb+ efflux assays are used as a measure of K+ channel function (Rezazadeh, 2004; Terstappen, 1999). The Rb+ assay is based on the ability of K+ channels to conduct Rb+ in a similar fashion to K+ (Hille, 1992) as the chemical properties of Rb+ are similar to those of K+. The Rb+ assay involves incubation of cells with Rb+ for a number of hours as it is taken up by the cells via the Na+-K+ ATPase (Gill, 2004). Opening of K+ channels allows the efflux of accumulated Rb+ from the cells (Cheng, 2002; Tang, 2001). Levels of Rb+ in the supernatant following channel activation are an indicator of channel function (Terstappen, 1999). To date, the use of the Rb+ assay for measuring K+ channel function has been successful for the KCNQ K+ channels (Jow, 2006), Ca2+-activated K+ channels (Parihar, 2003) and the HERG K+ channel (Chaudhary, 2006; Cheng, 2002).
In the present study the Rb⁺ assay was developed to test the effect of drugs on the HERG K⁺ channel in HERG transfected HEK293 cells. A change in the membrane potential leads to the activation of HERG. High K⁺ solution depolarizes cells by reversing the K⁺ equilibrium potential (Arner, 1981; Cheng, 2002). In this study, high K⁺ solution caused a significant increase in Rb⁺ efflux in HERG transfected HEK293 cells and had no significant effect in wild HEK293 cells. Rb⁺ was measured by atomic absorption spectroscopy and the K⁺-evoked efflux obtained was comparable to efflux obtained in other studies (Chaudhary, 2006; Rezazadeh, 2004). Efflux was in the range of 55 - 65 % of total load after depolarization over a 10 min period in solution containing K⁺ at concentrations higher than 50 mM, this value is similar to that obtained in HERG transfected CHO cells (Cheng, 2002). A time-dependent increase in Rb⁺ efflux was observed in cells after addition of K⁺ at 5.4, 25 and 50 mM and this was inhibited by the known HERG inhibitor astemizole (Suessbrich, 1996a), which reduced the efflux to the values obtained in basal solution (K⁺, 5.4 mM). Similar results were previously reported with the potent HERG inhibitor E-4031 (Zhou, 1998a) in HERG transfected CHO cells (Cheng, 2002). These results suggest that basal efflux is not due to HERG since native HEK293 cells not containing HERG had basal efflux values that were comparable to those obtained in HERG transfected HEK293 cells and astemizole-treated cells after K⁺ depolarization. The HEK293 cell line contains an endogenous transient outward current (Zhou, 1998a), and this most likely accounts for the background Rb⁺ efflux.

Terfenadine, another HERG inhibitor (Roy, 1996) was tested in the present study at various concentrations which inhibited Rb⁺-induced efflux in HERG transfected
HEK293 cells in high K⁺ solution but had no effect on efflux in native HEK293. The standard operating procedure developed using K⁺ (54 mM) solution were used to induce Rb⁺ efflux over a 10 min period, as this concentration showed the shortest duration with most effect. K⁺-depolarization does not have the same characteristics as action potential depolarization which is transient and of varying potentials. As such, K⁺-depolarization is a tool rather than a physiologically relevant stimulus.

2.5.7 Effect of drugs on Rb⁺ efflux

There are many identified K⁺ channels in a variety of cell types (see section 1.1.2.3) which are known to be blocked by pharmacological agents. HERG transfected HEK293 cells provide an opportunity to determine whether these agents are selective for their non-HERG channels or whether they additionally block HERG. This latter point is important because when native cells are used which constitutively express HERG, other K⁺ channels may also be present and HERG effects can only be resolved when the non HERG channels are blocked pharmacologically.

4-Aminopyridine blocks a range of K⁺ channels including A-type K⁺ channels (Rogawski, 1985), dorsal root ganglion K⁺ channels (Stansfeld, 1986) and other voltage-gated K⁺ channels (Grissmer, 1994) in the range of 1-2000 µM. In the present study, 4-AP only had a small effect on HERG K⁺-induced Rb⁺ efflux (< 10 % inhibition at 30 µM, the highest concentration tested). It may be that the concentration tested was not high enough to detect appreciable effects as in another study 4-AP had an IC₅₀ of 4.4 mM in HERG transfected HEK293 cells using the patch clamp technique (Ridley, 2003). It has also been reported that HERG inhibition by 4-
AP is accompanied by prolongation in the action potential duration in guinea pig ventricles (Ridley, 2003). It seems likely from these results that 4-AP does block the HERG encoded K+ channel at high concentrations.

Ba$^{2+}$ blocks a range of K+ channels including the delayed rectifier K+ channels (Taglialatela, 1993b). In the present study, Ba$^{2+}$ was tested up to 30 µM and it had only a small facilitatory effect. Ba$^{2+}$ blocks HERG currents expressed in Xenopus oocytes using the patch clamp technique at concentration above 1 mM (Weerapura, 2000). This inhibition of HERG is state-dependent and only occurs at voltages between -40 and 0 mV and is not observed at higher voltages. The lack of HERG inhibition by Ba$^{2+}$ in the present study may be due to the low concentration tested.

Cd$^{2+}$ blocks voltage activated K+ channels such as the slow delayed rectifier channels in Xenopus oocytes (Chen, 2003) and delayed rectifier K+ channels in guinea pig ventricular myocytes (Daleau, 1997). Cd$^{2+}$ (500 µM) also causes changes to HERG functional properties closely resembling channel inhibition in Xenopus oocytes using the patch clamp technique (Fernandez, 2005). Cd$^{2+}$ slows the rate of channel activation and accelerates the rate of channel inactivation. The results obtained in the current study showed that Cd$^{2+}$ decreased K+-induced Rb+ efflux in the range of 10 nM - 300 µM, which is in accordance with known Cd$^{2+}$ effect on HERG.

TEA had a small facilitatory effect on K+-induced Rb+ efflux when tested using the Rb+ assay. It has been suggested that TEA causes changes to HERG activation kinetics by binding to the HERG channel and impeding inactivation (Shimizu, 2003).
This could explain the small facilitatory effect observed using the Rb\(^+\) assay in the current study. Importantly TEA did not inhibit HERG even though it has been reported to block various K\(^+\) channels including dorsal root ganglion K\(^+\) channels (Hille, 1967) and delayed rectifier channels (see Table 1-1).

To validate the animal-free HERG transfected HEK293 cell line of the present study, several known HERG K\(^+\) channel inhibitors were tested including terfenadine, astemizole, pimozide, quinidine sulphate, and haloperidol (see section 1.4). The inhibitory effect of these drugs on HERG was determined by calculating the half maximal inhibition (IC\(_{50}\)) of K\(^+\)-induced Rb\(^+\) efflux since Rb\(^+\) efflux is an indicator of channel function (Daniel, 1991).

In the present study in HERG transfected HEK293 cells using K\(^+\) depolarization with Rb\(^+\) efflux and patch clamp electrophysiology, terfenadine had an inhibitory effect on HERG activity. An IC\(_{50}\) value for the Rb\(^+\) efflux was 260 nM which was similar to HERG transfected HEK293 cells (204 nM) measured by patch clamp (Crumb, 2000). This data shows that terfenadine is more potent in HEK293 cells than that reported in CHO cells (1800 nM) using the Rb\(^+\) assay (Tang, 2001). CHO cells are of animal origin and seem to often give variant results (see Table 2-4). Interestingly terfenadine was less potent in the study of Dorn (2005) who used a fluorescent potentiometric dye in HERG transfected CHO cells resulting in an IC\(_{50}\) value of 794 nM. This may be due to the fact that the dye does not measure channel activity directly but rather the endpoint of membrane potential which may be influenced by several factors including establishment of membrane potential by the dyes themselves or calibration.
of the dye fluorescence (Smith, 1982). In experimental models terfenadine increased the QT interval in guinea pig isolated hearts (Pinney, 1995b) and prolonged the action potential duration (Ki, 1996) at concentrations in the range of 0.01 – 10 µM. However, it had no affect on dogs at 10 µM (Gintant, 2001). In man, terfenadine has been reported to cause prolongation in the QT interval at a concentration of 200 nM which may occur in the presence of drugs which inhibit cytochrome P450 (Honig, 1993) and therefore impede terfenadine metabolism. Thus drug-drug interactions may be very important.

The IC_{50} value for astemizole in the present study was very similar to other reported studies where no differences in potency was observed between CHO and HEK293 cells (Cheng, 2002; Rezazadeh, 2004; Tang, 2001), nor with the different assay conditions of patch clamp (Zhou, 1999), Rb^+ efflux (Rezazadeh, 2004) and fluorescent dye (Dorn, 2005). In experimental models such as dogs, astemizole increases the action potential duration (Weissenburger, 1999). In man, astemizole increases the QT interval at a plasma unbound concentration of 0.2 µM (Paton, 1985; Simons, 1988). This is well above the therapeutic unbound plasma levels which fall within the range of 0.20 – 0.26 (Gilman, 1990).

Pimozide, a neuroleptic drug reduced K^+-induced Rb^+ efflux with an IC_{50} of 38.7 nM in the current study. This value is 2-fold higher than that reported for pimozide inhibition of HERG using the patch clamp technique in HERG transfected CHO cells (Kang, 2000; Kongsamut, 2002). Compared to other assays, the IC_{50} value of pimozide obtained in the current study was 130-fold lower than that obtained using a
fluorescent assay in HERG transfected CHO cells (Dorn, 2005). Pimozide has been reported to increase the QT interval in human at a concentration of 0.3 µM (Fulop, 1987), which does not fall within the therapeutic unbound plasma level of 0.09 – 0.43 nM (Gilman, 1990) and even 1 nM which has been observed in the presence of CYP3A4 inhibitors (Desta, 1999). This calls into question a link between HERG inhibition by pimozide and prolongation in the QT interval.

In the present study haloperidol, an antipsychotic drug reduced K+-induced Rb+ efflux with an IC50 of 417 nM. This value is 2-fold higher than that obtained in HERG transfected HEK293 cells using the patch clamp technique (Diaz, 2004), whilst in Xenopus oocytes, haloperidol resulted in an IC50 value of 1 µM (Suessbrich, 1997). In comparison to other testing assays the fluorescent assay used by (Dorn, 2005) in HERG transfected CHO cells, resulted in an IC50 value 200-fold higher than that obtained in the present study. Haloperidol has been reported to increase the QT interval at a concentration of 3 µM (Henderson, 1991) which is well above the therapeutic unbound plasma levels of 1.2 – 3.6 nM (Gilman, 1990) suggesting that its cardiac toxicity is not necessarily linked to HERG inhibition as seen in vitro.

The results of the Rb+ efflux showed that amitriptyline, a psychotic drug reduced K+-induced Rb+ efflux and resulted in an IC50 of 1 µM. This value is less than 1.5-fold higher than that reported for amitriptyline inhibition of HERG using the patch clamp technique in HERG transfected HEK293 cells (Tie, 2000b). Amitriptyline caused a reduction of HERG currents in CHO cells by 34 % at 3 µM (Teschemacher, 1999) and resulted in an IC50 value of 23.0 µM in HERG expressed in Xenopus oocytes at a
voltage of -30 mV (Jo, 2000). Using a fluorescent assay, amitriptyline resulted in an IC₅₀ close to 20 µM (Dorn, 2005). In experimental animals, amitriptyline increases the action potential duration in dogs at a concentration range of 1 – 10 µM (Nishimoto, 1994), however it decreases the action potential duration at 20 µM (Wyse, 1996). In human, amitriptyline has been associated with an increase in heart rate, however it had no effect on the QT interval (Veith, 1982).

Known weak inhibitors of the HERG K⁺ channel with reported IC₅₀ values above 10 µM had no effect on K⁺-evoked Rb⁺ efflux in HERG transfected HEK293 cells. These drugs included desipramine, spironolactone and diltiazem (Caballero, 2003; Dorn, 2005; Zhang, 1999). This may be expected as there was a tendency for the IC₅₀ in the present study using the Rb⁺ assay to be higher than those in the literature using the patch clamp technique. Hence, the IC₅₀ values for those weak inhibitors would have been expected to be well above the maximum tested concentration of 30 µM when tested using the Rb⁺ assay as a tendency to inhibit was apparent at this concentration for each of these drugs.

In the present study the antidepressant desipramine showed only a slight inhibitory effect on K⁺-induced Rb⁺ efflux at 30 µM whereas in previous studies it has been reported to have an IC₅₀ of 1.39 µM in HERG transfected HEK 293 cells using patch clamp technique (Ekins, 2002). This reinforces the finding that the Rb⁺ assay appears to show less potency on HERG than patch clamp. In experimental animals such as dogs desipramine caused a reduction in the duration of the action potential at a concentration of 1 µM (Muir, 1982). The therapeutic unbound plasma levels are in the
range of 27 - 108 nM (Gilman, 1990). This suggests that HERG activity observed in HERG transfected HEK293 cells is not within the therapeutic unbound plasma levels. In general desipramine at therapeutic levels does not cause prolongation of the QT interval. Even in cases of cardiac deaths with desipramine, it is unclear whether prolongation of the QT interval is responsible as other effects of desipramine or other confounding factors may be responsible (Johnson, 1996; Riddle, 1993).

Similarly to desipramine, the antihypertensive drug diltiazem showed a slight inhibitory effect on K⁺-induced Rb⁺ efflux at 30 µM. In previously reported studies, the IC₅₀ value in HERG transfected HEK293 cells was 17.3 µM (Zhang, 1999). This value is well above the therapeutic unbound plasma concentration which falls in the range of 53 – 122 nM (Gilman, 1990). In experimental animals, diltiazem caused a decrease in the action potential duration in guinea pigs at 10 µM (Gjini, 1998). To date, there are no cases reporting diltiazem’s cardiac toxicity or sudden death.

Two forms of quinidine were tested in this study, the commonly used quinidine sulfate and quinidine anhydrous. It was interesting to observe that quinidine anhydrous had a less potent effect on K⁺-induced Rb⁺ efflux compared to quinidine sulfate. The reported IC₅₀ value for quinidine sulfate using patch clamp is 400 nM (Paul, 2002), which is 9-fold more potent than that obtained in this study. The sulfate portion of the drug could predict the channel’s higher sensitivity to this form of the drug compared to the anhydrous form (see Figure 2-2). In experimental models, quinidine caused an increase in the action potential duration of guinea pig ventricular myocytes at concentrations between 1 – 10 µM (Komeichi, 1990). In man,
quinidine prolongs the QT interval at concentrations between 0.56 – 2.3 µM (Benton, 2000) which fall within the therapeutic unbound plasma levels of 924 – 3237 nM (Gilman, 1990). This suggests a link between HERG inhibition and a prolongation in the QT interval by quinidine. However, quinidine also affects other cardiac ion channels which may complicate the analysis.

The above discussion focussed on individual drugs, however, it is important to analyse group data. It was decided to compare the K⁺-induced Rb⁺ efflux in the present animal-free HERG transfected HEK293 cells with patch clamp data for normal (animal assisted) HERG transfected HEK293, which is currently used for HERG testing in a variety of settings. The key issue perhaps is not the cell line per se as the characteristics of the present study shows the HERG transfected cells having properties matching those reported in the literature for animal-assisted HERG transfected HEK293 (see section 2.5.5). The key issue is the use of K⁺ depolarization with the Rb⁺ efflux assay. Clearly K⁺ depolarization for 10 min is not equivalent to the duration of depolarization produced by an action potential nor equivalent to the seconds of depolarization produced by patch clamp electrophysiology. However, the question is whether as an assay it can predict accurately inhibitors rather than physiologic relevance.

The first comparison is whether the weak inhibitors were picked up. The design which tested all compounds up to 30 µM did not allow for a strict comparison. Both diltiazem and spironolactone showed no sign of inhibition in the K⁺-induced Rb⁺ efflux in the present study even though in patch clamp they had an IC₅₀ of 17 and 23
µM respectively (Caballero, 2003; Dorn, 2005; Zhang, 1999). Further desipramine potency appears underestimated by the Rb⁺ efflux system by a factor of at least 1 log unit of the present study, although an inhibitory effect was becoming apparent at 30 µM. Higher concentrations should have been tested, however it needs to be noted that the therapeutic levels is in the range of 40 – 160 nM (Gilman, 1990).

Despite these results, when drugs in the moderate activity level were compared there was a very good correlation between patch clamp HERG transfected HEK293 cells and the Rb⁺ assay of the present study with a tendency of an underestimation of potency.

In comparing Rb⁺ assay between animal aided and animal-free HERG transfected HEK293 cells only two drugs can be compared with literature data, terfenadine and astemizole. The IC₅₀ values of 198 nM (Rezazadeh, 2004) versus 260 nM in the present study for terfenadine and 65 nM (Rezazadeh, 2004) versus 62 nM in the present study for astemizole. This shows that differences from patch clamp may be due to the Rb⁺ assay system rather than cell system. The Rb⁺ assay could be causing an underestimated value of drug potency due to the depolarization tool.

The potency of drugs tested were correlated with those obtained using the patch clamp technique from the literature. High K⁺ solution was used to depolarize the cells in this study compared to voltage clamp which allows for voltage clamp that has better control over the channel activation (Hille, 1992). The patch clamp technique is considered the gold standard for studying ion channel function as it
provides direct measurement of currents under controlled conditions. The assay provided good correlation with patch clamp data even though the depolarization conditions differed. The data obtained were of underestimated potency however a good linear relationship across a wide range of drugs was observed. This indicates that the Rb$^+$ assay is able to predict HERG inhibition and reliably rank the drugs order of potency. Using the Rb$^+$ assay, 70 % of the tested compounds had an IC$_{50}$ below 30 µM, the maximum concentration tested. This proportion of active drugs are in agreement with those obtained using a fluorescent membrane potential assay (Dorn, 2005). The drugs that did not result in IC$_{50}$ values were the known weak HERG inhibitors and it is expected that the value would be above the maximum concentration used in the assay. The key question is whether the assay is robust enough for clinical prediction. In terms of clinical reports whilst there are strong links in many cases between sudden death or torsadogenic propensity, QT interval prolongation and HERG inhibition (Shah, 2002a; Shah, 2002b; Shah, 2002c), in many cases the data is not clear cut particularly for drugs with weak HERG activity and those which have other pharmacological actions (Johnson, 1996; Riddle, 1993). In many cases, drugs have HERG activity in patch clamp studies only at concentrations far greater than therapeutic levels. The strong correlation in the present study with patch clamp data suggests that the assay system developed is useful but that it needs to be calibrated back to patch clamp data for safety evaluation. In particular weak inhibitors need to be tested at far higher concentrations than in the present study.

In conclusion, the development of a HERG transfected HEK293 cells using animal-free methods was successful. This study provides potential tools for gene insertion
into cells eliminating the use of animal-derived reagents. The system developed resulted in data for drug testing of similarity to those reported in the literature.
Chapter 3: The use of a neuroblastoma cell line to predict the inhibitory effect of drugs on HERG.

3.1 Summary:

1. Constitutively HERG expressing cell line SH-SY5Y was used to determine the effect of drugs on the HERG K+ channel.

2. Using specific HERG primers, the RNA in the neuroblastoma cells was reverse-transcribed and amplified using PCR. Bands at an expected size of 575 bp in the neuroblastoma cells confirmed HERG presence showing the same band detected in HERG transfected HEK293 cells.

3. K+-induced Rb+ release from the cells was used to determine the level of K+ channel activity. The maximum efflux of Rb+ reached 30 % of total cell content in the presence of 50 mM K+.

4. SH-SY5Y cells contain multiple K+ channels. A cocktail of various K+ channel inhibitors were tested to determine which channels contributed to the K+-induced Rb+ efflux to isolate efflux caused by the HERG K+ channel. Apamin and charybdotoxin did not affect the Rb+ efflux. 4-Aminopyridine, atropine, Ba2+ and tetraethylammonium significantly decreased K+-induced Rb+ efflux. However, it is unclear whether this indicates non-HERG channels since HERG may be inhibited directly by some of these agents (see section 2.4.6.3).
5. Tetraethylammonium (TEA) was the most effective inhibitor of K+-induced Rb+ efflux in SH-SY5Y cells. TEA had no effect on HERG even at concentrations as high as 10 mM when tested using the transfected HEK293 cells which suggests that it might be useful to isolate HERG effects (see section 2.4.6.3.1).

6. Known HERG inhibitors of different classes: terfenadine, astemizole, haloperidol, amitriptyline, diltiazem, pimozide, quinidine, desipramine and spironolactone were tested on K+-induced Rb+ efflux in the presence of TEA (10 mM). The IC$_{50}$ values of these drugs were obtained from concentration-response curves fitted with the Hill equation. The IC$_{50}$ values obtained using the Rb+ assay were compared to those obtained from patch clamp studies in HERG transfected HEK293 cells in the literature and were found to be well correlated with a correlation coefficient value of 0.82.
3.2 Introduction

3.2.1 Choice of cell line

Many mammalian cell types express the HERG K⁺ channel including cardiac cells, neurons, smooth cells and cancer cells (see section 1.5). The human neuroblastoma cell line-SH-SY5Y has been found to closely resemble neurons and expresses the HERG gene and has functional HERG K⁺ channels (Arcangeli, 1999). This cell line is an immortalized cell line that expresses various ion channels and was chosen as it represents an excitable system. No immortalized cardiomyocyte cell lines are available yet Primary cardiomyocyte cells lose their characteristics after two days in culture and different batches would be required. Therefore the work involved and the consistency between batches would be an issue in addition to resolving HERG effects from other currents.

3.2.2 Characteristics of the SH-SY5Y cell line

The human neuroblastoma SH-SY5Y cells were derived from a parental SK-N-SH cell line, originally cloned from a metastatic bone tumour in 1970 (Biedler, 1973; Ross, 1983a). The SK-N-SH cell line was cloned three times to produce SH-SY, SH-SY5 and then finally to produce the SH-SY5Y cell line. The parental cell line displays both epithelial as well as neuroblast cells, and the SH-SY5Y cells display neuroblast-like cells with short dendrites. This property of the cells is lost after passage 20. SH-SY5Y cells are electrically excitable and constitutively express many different voltage-gated ion channels such as A currents (Forsythe, 1992), slow and rapid delayed rectifier K⁺ currents (Reeve, 1992), inward rectifier I\textsubscript{IR} (Arcangeli, 1995b), Ca²⁺-sensitive K⁺...
currents, M currents, tetrodotoxin-sensitive Na\(^+\) channels, L- and N-type Ca\(^{2+}\) channels (Forsythe, 1992; Morton, 1992; Reeve, 1992; Reeve, 1994; Seward, 1990). Ligand-gated channels were also detected including nicotinic acetylcholine receptors (Gould, 1992). The ion channels are well characterized pharmacologically. Patch clamp recordings of action potentials from these cells resembles fast excitation of sympathetic neuronal characteristics (Mathie, 1987) and the cells closely resemble a human foetal sympathetic ganglion cells with morphological and biochemical similarities (Hervonen 1978).

In SH-SY5Y cells, depolarization is terminated by the opening of K\(^+\) channels which allows intracellular K\(^+\) to exit and therefore repolarizes the cells (see section 1.1.3). In clones of the parent cell line, subpopulations of cells have been discovered where the HERG encoded channel has been identified as the predominant K\(^+\) channel responsible for the hyperpolarization phase such as the subclone 21S (D'Amico, 2003).

Normal populations of SH-SY5Y cells have multiple K\(^+\) channels making selective actions on HERG difficult to assess. Patch clamp electrophysiology can to some extent isolate HERG currents by manipulation of the activation characteristics as has been reported (Taglialatela, 1998). However, if the Rb\(^+\) efflux assay is to be used multiple K\(^+\) channels could be a potential problem as Rb\(^+\) could transit by any K\(^+\) channel. The Rb\(^+\) assay nevertheless has many potential advantages over patch clamp in that it can be used for automated analyses and medium throughput. In order to deal with the problem of multiple K\(^+\) channels with the Rb\(^+\) assay, the aim of
the present study was to block the potential non-HERG K+ channels pharmacologically to potentially reveal pure HERG effects; and then test a range of HERG inhibitors and compare the results to literature data in HERG transfected HEK293 cells. SH-SY5Y cells were cultured using non-animal methodologies.
3.3 Methods

3.3.1 Culture of SH-SY5Y

The human neuroblastoma cell line (SH-SY5Y) was obtained from the European Collection of Cell Culture (ECACC) at passage 15. Cells were grown in the presence of non-animal reagents. Cells were cultured in growth medium containing RPMI containing L-glutamine (5 mM), non-essential amino acids (1 % v/v), gentamicin (15 \( \mu g/ml \)) and human serum (10 % v/v). Cells were maintained at 37 °C and 5 % CO\(_2\) sterile incubator. Cells were used only up to passage 20 as they lose their neuronal characteristics after passage 20. Cell detachment from polystyrene plastic ware (Nunc easy flasks, In Vitro Inc, Melbourne, Australia) was done by the addition of TrypLE Select, a bacterial dissociation enzyme. Cells were passaged by the addition of 1 ml TrypLE Select for 5min at room temperature; the cells were then resuspended in 5 ml RPMI and spun at 100 g for 5 min at room temperature. The pellet was then resuspended in 5 ml growth medium and 1 ml of suspension was added to 5 x 75 cm\(^2\) culture flasks. Cells were stored in growth medium containing DMSO (10 %) in liquid nitrogen.

Cells were also cultured under reduced serum conditions using Advanced RPMI containing human serum (2 % v/v). Advanced RPMI is a growth medium containing the same ingredients as a standard RPMI with additional ingredients allowing for cell growth under reduced serum conditions. These ingredients include non essential amino acids, ethanolamine, glutathione, ascorbic acid phosphate, recombinant insulin, magnesium chloride, cupric sulfate and ammonium metavanadate (GIBCO-Invitrogen, 2004a).
3.3.2 Polymerase chain reaction (PCR)
Protocol as per section 2.3.4.

3.3.3 Rubidium efflux assay
The rubidium assay was used to test whether the SH-SY5Y cells were a suitable tool for drug safety testing. Assay conditions such as loading and depolarization were applied as per section 2.3.6 to allow for assay comparison under the same conditions.

3.3.3.1 Assay conditions

3.3.3.1.1 Cell count
Protocol as per section 2.3.6.1.1

3.3.3.1.2 Rubidium efflux assay
Protocol as per section 2.3.6.1.2

3.3.3.1.3 Rubidium determination using atomic absorption spectroscopy
Protocol as per section 2.3.6.1.3.

3.3.4 Statistics and calculations
All data were expressed as the mean and standard error of the mean (SEM) with the number of observations (n) being given. Data calculation, IC$_{50}$ calculations and
graphical presentations were completed in GraphPad Prism 4 software package (GraphPad software, Inc, San Diego, CA).

The IC$_{50}$ value of drugs is defined as the concentration required to reduce the K$^+$-induced Rb$^+$ efflux to 50 % of the maximum efflux.

3.3.5 Materials, drugs and chemicals

All drugs and chemicals were purchased as per 2.3.9. Human serum (from clotted human male whole blood, Type AB, sterile-filtered, (Mycoplasma tested, virus tested) purchased from Sigma Aldrich (St Louis, USA). TrypLE Select, RPMI 1640 Medium was purchased from Invitrogen Corporation (Carlsbad, USA). L-glutamine and non-essential amino acids purchased from DKSH Australia Pty Ltd (Melbourne, Australia).
3.4 Results

3.4.1 Culture of SH-SY5Y

The cells were cultured in standard RPMI and human serum (10 %), as per section 2.3.2 and 3.3.1. The cells grew in an adherent monolayer and no coating material was required during culture. Growth medium was exchanged every 2 days and cells were passaged at 80 % confluence using TrypLE Select. The cell morphology is neuroblast-like, the cells did not form clumps in culture (Figure 3-1).

When cells were cultured in advanced RPMI, a growth medium developed to allow culture in reduced serum concentration, the cells showed signs of differentiations and long dendrites were developing (Figure 3-1). This effect was most evident when cells were serum-starved; hence, the cells were cultured using standard RPMI containing human serum (10 %) to avoid cell differentiation.
Figure 3-1: SH-SY5Y culture using animal-free reagents

Panel A: SH-SY5Y cells cultured in growth medium containing human serum (10 %), x 20 magnification. Panel B: SH-SY5Y cells cultured in growth medium containing human serum (10 %), x 60 magnification. Panel C: SH-SY5Y cells incubated in advanced RPMI medium with human serum (2 %), at 20 x magnification. Panel D: SH-SY5Y cells incubated in advanced RPMI medium with human serum (2 %), x 60 magnification.
3.4.2 PCR

PCR experiments were completed using specific HERG primers. PCR of the SH-SY5Y cells both showed the same band seen for the transfected HEK293 cells at 575 base pairs (Figure 3-2). This band was absent in the native HEK293 cells. As a positive control for HERG, the plasmid used for HERG transfection was amplified by PCR; the amplified DNA had the same size as the HERG transfected DNA in HEK293 cells.
Figure 3-2: HERG expression in HEK293 and neuroblastoma cell lines.

PCR products were amplified as per protocol using specific HERG primers (see Methods). The resulting PCR products were electrophoresed on a 0.7 % agarose gel at 70 V for 60 min. Lane 1, 1 Kb DNA ladder, lane 2, HEK293 cells, lane 3, HERG-transfected HEK 293 cells, lane 4, HERG plasmid in pcDNA3.1, lane 5, SH-SY5Y cells.
3.4.3 Rubidium assay

The Rb⁺ efflux assay was used to measure K⁺ channel activity. Experiments were conducted to determine the characteristics of Rb⁺ uptake and release in SH-SY5Y. Incubation of cells with RbCl (5.4 mM) for 180 min resulted in Rb⁺ accumulation in SH-SY5Y cells. The concentration of Rb⁺ in the intracellular solution was 11.5 ± 1.13 mg/L on average (n = 35) in a well containing 1x10⁵ cells lysed in 500 μl lysis buffer. Depolarization of cells by the addition of high K⁺ (50 mM) caused a significant increase in the Rb⁺ efflux (30 %) compared to cells containing wash buffer (5 mM K⁺) (Figure 3-3).
Figure 3-3: Effect of K⁺ on Rb⁺ efflux

Rb⁺ release in SH-SY5Y cells incubated with wash buffer (basal, 5 mM K⁺) and in SH-SY5Y cells incubated with high K⁺ buffer (50 mM K⁺) for 10 min. The percentage efflux represents Rb⁺ release as a % of total cellular Rb⁺. Data represents mean ± SEM (n = 6 - 8). * Denotes significantly different from basal (P < 0.05, Student t-test).
3.4.3.1 Inhibition of Rb\(^+\) efflux by K\(^+\) channel blockers

Several classical K\(^+\) channel blockers were tested to determine whether K\(^+\) induced Rb\(^+\) efflux caused by HERG activation could be isolated from other K\(^+\) channels expressed in SH-SY5Y cells.

This range of classical inhibitors was also tested in HERG transfected HEK293 cells (see section 2.4.6.3). The HERG transfected HEK293 cells only contain HERG channels (Zhou, 1998a) and this was an indicator whether the classical inhibitors inhibited HERG in addition to their recognized K\(^+\) channel.

3.4.3.1.1 Atropine

Atropine inhibits muscarinic receptors and prevents the activation of M-receptors linked K\(^+\) channels if these are activated by acetylcholine (Koumi, 1997). When tested in SH-SY5Y cells atropine (0.01 mM) slightly, but significantly reduced the K\(^+\)-induced Rb\(^+\) efflux (Figure 3-4) indicating either that M receptor linked channels are active or that atropine may affect HERG. In HERG transfected HEK293 cells atropine had a small inhibitory effect on K\(^+\)-induced Rb\(^+\) efflux (see section 2.4.6.3.3). Since these cells do not express M linked K\(^+\) channels, the results indicate that atropine may affect HERG and is not suitable as a pharmacological intervention to isolate HERG mediated Rb\(^+\) efflux.
3.4.3.1.2 4-aminopyridine

4-Aminopyridine (4-AP) (2 mM), an inhibitor of voltage-gated K+ channels (Table 1-1) had a significant inhibitory effect on K+ induced Rb+ efflux in SH-SY5Y cells (Figure 3-4). In HERG transfected HEK293 cells a low concentration of 4-AP (30 µM) had a small inhibitory effect on K+-induced Rb+ efflux (see section 2.4.6.3.4). The results indicate that 4-AP may affect HERG and may not be suitable as a pharmacological intervention to isolate HERG mediated Rb+ efflux.

3.4.3.1.3 Ba2+

Ba2+ inhibits M-currents in sympathetic neurons (Kotani, 2000) and blocks delayed K+ channels (DRK) (Taglialatela, 1993a). When tested (20 mM) in SH-SY5Y cells it significantly reduced the K+-induced Rb+ efflux (Figure 3-4). In HERG transfected HEK293 cells Ba2+ (30 µM) enhanced K+-induced Rb+ efflux (see section 2.4.6.3.2).

3.4.3.1.4 Apamin

Apamin (300 nM), an inhibitor of small Ca2+ activated K+ channels (Stocker, 2004) had no effect on K+-induced Rb+ efflux (Figure 3-4). No studies to date have provided evidence of the expression of these channels in SH-SY5Y cells. Therefore they probably are not active under the assay conditions.

3.4.3.1.5 Charybdotoxin

Charybdotoxin (50 nM), an inhibitor of large conductance Ca2+ activated K+ channels (Garcia, 1995) had a no effect on K+ induced Rb+ efflux (Figure 3-4). No studies to
date have provided evidence of the expression of these channels in SH-SY5Y cells. Therefore they probably are not active under the assay conditions.

3.4.3.1.6 **Tetraethylammonium**

Tetraethylammonium (TEA), an inhibitor of ATP-sensitive channels, voltage-gated K⁺ channels, KCNQ channels and Ca²⁺ activated K⁺ channels had a significant inhibitory effect on K⁺ induced Rb⁺ efflux (Figure 3-4). However, in HERG transfected HEK293 TEA had no effect on K⁺-induced Rb⁺ efflux (see section 2.4.6.3.2). The results indicate that TEA is acting on K⁺ channels other than HERG in SH-SY5Y cells. Therefore TEA may be suitable as a pharmacological intervention to isolate HERG mediated Rb⁺ efflux.

To further confirm the effect of TEA on K⁺ channel activity in these cells, TEA was tested in the presence of a potent HERG blocker, pimozide (Kang, 2000). TEA (10 mM) significantly reduced the K⁺-induced Rb⁺ efflux by 8 % (Figure 3-5). The HERG inhibitor pimozide (10 µM) reduced the K⁺-induced Rb⁺ efflux by 22%, but did not abolish it. The addition of TEA and pimozide together abolished the Rb⁺ efflux. To examine the possibility of any effect of TEA on HERG, 3 concentrations of the TEA were tested in the presence and the absence of a maximum concentration of pimozide. All TEA concentrations caused a significant decrease in efflux in the presence of pimozide (Figure 3-5). This indicates that TEA has an independent action to pimozide.
Figure 3-4: Effect of various K⁺ channels blockers on K⁺-induced Rb⁺ efflux in SH-SY5Y cells over a 10-min incubation period.

Rb⁺ release was measured in the presence of 50 mM K⁺ in all experiments. Ba²⁺ (20 mM), atropine (0.01 mM), 4-aminopyridine (2 mM) and TEA (10 mM) were present in some experiments. The percentage efflux represents Rb⁺ release as a % of total cellular Rb⁺. Data represents mean efflux ± SEM, (n = 3 - 9). * Denotes significantly different to control (P < 0.05, unpaired Student’s t-test). AT is atropine, CHAR is charybdoxin, AP is apamin.
Figure 3-5: Effect of various TEA concentrations and pimozide on K⁺-induced Rb⁺ efflux.

Rb⁺ release was measured in the presence of 50 mM K⁺ in all experiments. Effect of various concentrations of TEA on Rb⁺ efflux in SH-SY5Y cells in the presence and absence of the HERG blocker pimozide. The percentage efflux represents Rb⁺ release as a % of total cellular Rb⁺. Data represents mean efflux ± SEM, (n = 3 – 9). * Denotes significantly different compared to control (P < 0.05, Student’s t-test).
3.4.3.2 Effect of HERG inhibitors on K+-induced Rb+ efflux in the presence of TEA

Several known HERG inhibitors were tested in the SH-SY5Y neuroblastoma cell line. The drugs were tested in the presence of TEA (10 mM) and their IC$_{50}$ values were determined after constructing a concentration-response curve.

3.4.3.2.1 Pimozide

Pimozide (1 nM - 30 μM) concentration-dependently inhibited K$^+$ (50 mM) evoked Rb$^+$ efflux in SH-SY5Y cells (Figure 3-6). Pimozide was able to completely inhibit the K$^+$-induced Rb$^+$ efflux at 30µM (Figure 3-6). The IC$_{50}$ value for pimozide was 37.8 ± 0.2 nM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient ($r^2$) of 0.7112.

3.4.3.2.2 Haloperidol

Haloperidol (1 nM - 30 μM) concentration-dependently inhibited K$^+$ (50 mM) evoked Rb$^+$ efflux in SH-SY5Y cells (Figure 3-7). Haloperidol inhibited the K$^+$-induced Rb$^+$ efflux with a maximal inhibition of 100 % at 30μM (Figure 3-7). The IC$_{50}$ value for haloperidol was 105.00 ± 0.04 nM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient ($r^2$) of 0.9636.
3.4.3.2.3 Amitriptyline

Amitriptyline (1 nM – 30 μM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in SH-SY5Y cells (Figure 3-8). Amitriptyline had a moderate inhibitory effect on the K⁺-induced Rb⁺ efflux with a maximum inhibition of 40 % at 30 μM (Figure 3-8). The IC₅₀ value for amitriptyline was 13.34 ± 0.21 μM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.9877.

3.4.3.2.4 Quinidine anhydrous

Quinidine anhydrous (1nM – 30 μM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in SH-SY5Y cells (Figure 3-9). Quinidine anhydrous had a moderate inhibitory effect on the K⁺-induced Rb⁺ efflux with a maximum inhibition of 67 % at 30 μM (Figure 3-9). The IC₅₀ value for quinidine anhydrous was 16.34 ± 0.75 μM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.8996.

3.4.3.2.5 Quinidine sulfate

Quinidine sulfate (1 nM – 30 μM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in SH-SY5Y cells (Figure 3-10). Quinidine sulfate had a moderate inhibitory effect on the K⁺-induced Rb⁺ efflux with a maximum inhibition of 70 % at 30 μM (Figure 3-10). The IC₅₀ value for quinidine sulfate was 4.7 ± 0.14 μM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.8521.
3.4.3.2.6 Terfenadine

Terfenadine (1 nM – 30 µM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in SH-SY5Y cells (Figure 3-11). Terfenadine completely inhibited the K⁺-induced Rb⁺ efflux at 30 µM (Figure 3-11). The IC50 value for terfenadine was 215 ± 0.05 nM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.97.

3.4.3.2.7 Diltiazem

Diltiazem (1 nM – 30 µM) concentration-dependently inhibited K⁺ (50 mM) evoked Rb⁺ efflux in SH-SY5Y cells (Figure 3-12). Diltiazem was able to inhibit the K⁺-induced Rb⁺ efflux with a maximal inhibition of 76 % at 30 µM (Figure 3-12). The IC50 value for diltiazem was 15.57 ± 0.97 µM (Table 3-1) and was calculated after fitting the data using non-linear fit curve. The plotted curve had a fit coefficient (r²) of 0.89.

3.4.3.2.8 Desipramine

Desipramine (1 nM – 30 µM) had a slight but significant inhibitory effect on K⁺-induced Rb⁺ efflux (Figure 3-13). Desipramine was able to inhibit the K⁺-induced Rb⁺ efflux with a maximal inhibition of 30 % at 30 µM (Figure 3-13). No IC50 value was calculated as no curve was fitted and the IC50 value was referred to as > 30 µM.

3.4.3.2.9 Spironolactone

Spironolactone (1 nM – 30 µM) had a slight but significant inhibitory effect on K⁺-induced Rb⁺ efflux at concentrations between 3-30 µM (Figure 3-14). Spironolactone
was able to inhibit the K\(^+\)-induced Rb\(^+\) efflux with a maximal inhibition of 26 % at 30 µM (Figure 3-14). No IC\(_{50}\) value was calculated as no curve was fitted and the IC\(_{50}\) value was referred to as > 30 µM.
Figure 3-6: Pimozide concentration curve

Effect of pimozide on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 pimozide concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient (r²) of 0.7112. The IC₅₀ value obtained from the curve was 37.8 ± 0.2 nM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-7: Haloperidol concentration curve

Effect of haloperidol on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 haloperidol concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient ($r^2$) of 0.9636. The IC$_{50}$ value obtained from the curve was 105.00 ± 0.04 nM. * Denotes significantly different from 1 ($P < 0.05$, ANOVA, followed by Dunnett’s test).
Figure 3-8: Amitriptyline concentration curve

Effect of amitriptyline on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 amitriptyline concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient (r²) of 0.9877. The IC₅₀ value obtained from the curve was 13.336 ± 0.21 µM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-9: Quinidine anhydrous concentration curve

Effect of quinidine anhydrous on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 quinidine anhydrous concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient ($r^2$) of 0.8996. The IC$_{50}$ value obtained from the curve was 16.34 ± 0.75 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-10: Quinidine sulfate concentration curve

Effect of quinidine sulfate on K+-induced Rb+ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb+ efflux over a 10 min period in each well was expressed as a ratio of total Rb+ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K+-evoked Rb+ efflux in the presence of 10 quinidine sulfate concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient (r²) of 0.8521. The IC₅₀ value obtained from the curve was 4.7 ± 0.14 µM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Effect of terfenadine on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 terfenadine concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient (r²) of 0.97. The IC₅₀ value obtained from the curve was 215 ± 0.05 nM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-12: Diltiazem concentration curve

Effect of diltiazem on K⁺-induced Rb⁺ efflux in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 diltiazem concentrations was used to construct a concentration-response curve. The curve represents a non-linear curve fit with a fit coefficient ($r^2$) of 0.89. The IC₅₀ value obtained from the curve was 15.57 ± 0.97 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-13: Desipramine concentration curve

Effect of desipramine on K⁺-induced Rb⁺ in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 desipramine concentrations was used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 3-14: Spironolactone concentration curve

Effect of spironolactone on K⁺-induced Rb⁺ in the presence of TEA (10 mM) in SH-SY5Y neuroblastoma cells. Rb⁺ efflux over a 10 min period in each well was expressed as a ratio of total Rb⁺ content (supernatant and inside cells). All data were normalized to the efflux in the absence of drug. Each data point represents mean efflux from 6 wells ± SEM. K⁺-evoked Rb⁺ efflux in the presence of 10 spironolactone concentrations was used to construct a concentration-response curve. No IC₅₀ value could be calculated and it was represented as > 30 μM. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
3.4.3.3 **Comparison of Rb\(^+\) efflux and patch clamp studies**

Electrically stimulated HEK293 cells transfected with HERG generate a current carried by the outward flow of K\(^+\). This can be measured by patch clamp electrophysiology. In this section literature values for inhibition of HERG by drugs using the patch clamp technique were compared to those obtained using the K\(^+\) evoked Rb\(^+\) efflux in this study (Table 3-1). There was a strong relation for the drugs between both techniques with a correlation coefficient \((r^2)\) of 0.82 (Figure 3-15). The regression line had a slope of 0.92.

To determine whether the Rb\(^+\) assay predicts similar IC\(_{50}\) values of drugs using different systems, the results of the SH-SY5Y and HERG transfected HEK293 cells (see Chapter 2) were correlated (Figure 3-16). The regression line, \(y = 0.93x + 1.07\) resulted in a correlation coefficient \((r^2)\) of 0.92 for the 6 positive drugs tested in both cell lines.
Figure 3-15: Correlation of IC$_{50}$ values from Rb$^+$ assay and patch clamp

Correlation between IC$_{50}$ values obtained using patch clamped HERG transfected HEK293 cells and Rb$^+$ assay using SH-SY5Y cells. The data was fitted with a line of identity shown as a dashed line. The regression line is shown as a solid line. The regression line, $y = 0.92x + 1.09$ resulted in a correlation coefficient ($r^2$) of 0.82 for the 7 positive drugs tested.
Figure 3-16: Correlation of IC$_{50}$ values from Rb$^+$ assay in SH-SY5Y and HERG transfected HEK293 cells

Correlation between IC$_{50}$ values obtained using HERG transfected HEK293 cells and SH-SY5Y cells using the Rb$^+$ assay. The data was fitted with a line of identity shown as a dashed line. The regression line is shown as a solid line. The regression line, $y = 0.93x + 1.07$ resulted in a correlation coefficient ($r^2$) of 0.92 for the 6 positive drugs tested in both cell lines.
### Table 3-1: Comparison of IC$_{50}$ values obtained using the Rb$^+$ assay in SH-SY5Y cells and in HERG transfected HEK293 obtained using the patch clamp technique in literature.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Literature Ref</th>
<th>Experimental Ref</th>
<th>Literature IC$_{50}$ (nM)</th>
<th>Experimental IC$_{50}$ (nM)</th>
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</thead>
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<td>260</td>
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<td>6</td>
<td>&gt; 30000</td>
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</tr>
<tr>
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<td></td>
<td>5</td>
<td>3650</td>
</tr>
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<td>Diltiazem</td>
<td>17300 (Zhang, 1999)</td>
<td></td>
<td>8</td>
<td>&gt; 30000</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>23000 (Caballero, 2003)</td>
<td></td>
<td>9</td>
<td>&gt; 30000</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>500 Chapter 4</td>
<td></td>
<td></td>
<td>83500</td>
</tr>
<tr>
<td>Astemizole</td>
<td>1.2 (Wang, 2003)</td>
<td></td>
<td>1</td>
<td>62</td>
</tr>
</tbody>
</table>
3.5 Discussion

3.5.1 Culture of SH-SY5Y

The human neuroblastoma cell line SH-SY5Y is commonly cultured in the presence of fetal calf serum and either DMEM (Tagliatela, 1998) or RPMI 1640 (Arcangeli, 1999) as the growth medium. This study reports the culture of the cell line using human serum and RPMI 1640 as the growth medium providing all nutrients and amino acids required for cell division. Advanced RPMI, a growth medium manufactured to reduce the use of serum in culture was tested in the presence of human serum (2 %). This medium resulted in the differentiation of cells detected by a change in morphology. Differentiation of cells can be induced by serum starving the cells (Glick, 2000) and in this study, the morphology was similar to that seen in ethanol treated cells, which causes differentiation of SH-SY5Y cells (Muhlbauer, 2003). It has also been reported that serum elimination leads to differentiation and apoptosis of SH-SY5Y cells indicating the need for serum in cell growth as it contains growth factors required for cell proliferation (Macleod, 2001; Meghani, 1993). Culture of cells in RPMI 1640 and human serum (10%) did not cause cell differentiation and cells grew in adherent monolayers displaying morphology of epithelial-like cells similar to those cultured using fetal calf serum (Ross, 1983a). Hence, the cells were cultured in the presence of human serum (10 %) to promote cell proliferation and avoid cell differentiation which leads to cell death.

3.5.2 Molecular data

Using primers specific for HERG DNA (Arcangeli, 1998), a single band was detected in SH-SY5Y cells. The band detected was of similar size to that in the amplified
plasmid used for transfection in HERG transfected HEK293 cells. PCR was used as it provides a useful tool for RNA detection using animal-free reagents. The bands detected were similar to those previously reported (Arcangeli, 1998; Shoeb, 2003). The density of HERG DNA bands in SH-SY5Y was lower than those detected in the transfected HEK293. It has been reported previously that the density of HERG in SH-SY5Y cells is ~50 times less that in HERG transfected HEK293 cells (Taglialatela, 1998). It has been previously reported that HERG expression is low in SH-SY5Y cells (Bianchi, 1998) and may be induced by retinoic acid treatment (Arcangeli, 1998). Western blotting was not used to confirm the expression of the HERG protein as it requires the use of antibodies raised in animals; however the Rb+ efflux assay was used as an indicator of channel function. It has been previously reported that the SH-SY5Y cells express functional HERG K+ channels using the patch clamp technique (Crociani, 2003).

### 3.5.3 Rb+ assay

The present study aimed at investigating the possibility of using the human cell line SH-SY5Y that constitutively expresses the HERG K+ channel as a system for testing the effect of drugs on the HERG K+ channel. Several HERG inhibitors such as astemizole and terfenadine inhibited channel function in the SH-SY5Y cells using the patch clamp technique (Taglialatela, 1998; Taglialatela, 2001). A study by Toral (1994) investigated the use of a human neuroblastoma cell line (TE671) for the identification of voltage-gated K+ channel inhibitors using a radioactive Rb+ assay. The authors used various K+ concentrations between 40 - 100 mM to induce depolarization of the cells. Interestingly, the maximum K+-induced Rb+ efflux in their study was 25 % at a
K⁺ concentration of 40 mM, 32 % at a K⁺ concentration of 80 mM and 34 % at a K⁺ concentration of 100 mM, all detected after a 20-min incubation period. In the present study, the K⁺-induced Rb⁺ efflux reached a maximum of 30 % at a K⁺ concentration of 54 mM at a 10 min incubation period. In the current study, 54 mM K⁺ was used to test drugs effect under the same conditions carried in chapter 2 to eliminate any change in factors that might contribute to difference in results. The K⁺-induced Rb⁺ efflux properties reported for the SH-SY5Y cells in this study and the TE671 cells show differences in depolarization response in both neuroblastoma cells. The background Rb⁺ efflux caused by leak channels in this study was 10 times lower than that reported for the TE671 cell line (Toral, 1994). This indicates the presence of less leak K⁺ channels in SH-SY5Y compared to TE671 cells.

To determine non HERG K⁺-induced Rb⁺ efflux, various K⁺ channel blockers were tested as per section 2.4.6.4. The voltage gated, delayed rectifier and the muscarinic receptor linked K⁺ channels inhibitors 4-aminopyridine, Ba²⁺ and atropine respectively caused a significant reduction in K⁺-induced Rb⁺ efflux. The reduction caused by 4-aminopyridine and Ba²⁺ is consistent with results obtained by (Toral, 1994) when tested in TE671. This could indicate that the respective ion channels contribute to the Rb⁺ efflux in the SH-SY5Y cells since 4-AP has an inhibitory effect on outward K⁺ currents (Reeve, 1992). However, another possibility is that these drugs inhibited HERG channels. Indeed 4-AP and atropine reduced Rb⁺ efflux in HERG transfected HEK293 cells in the present study which supports this contention. It has also been previously reported that 4-AP inhibits HERG currents in HERG transfected HEK293 cells using the patch clamp technique (Ridley, 2003).
The Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel inhibitors charybdotoxin and apamin did not inhibit K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux probably due to low or no channel expression in SH-SY5Y. To date, Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents have been detected but not identified in SH-SY5Y cells (Smart, 1995).

Whilst TEA (10mM) significantly decreased K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux in this study, it had no effect on K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux in the study reported by (Toral, 1994) for TE671 cells. Authors explained this result as a lack of delayed rectifier channel expression in the TE671 cell line. In the present study, K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux reduction by TEA was not likely to be caused by HERG inhibition, as TEA had no effect on HERG K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux in HERG transfected HEK293 cells in Chapter 2. It has been previously reported that TEA inhibited outward K\textsuperscript{+} currents that were not affected by 4-AP in SH-SY5Y cells (Reeve, 1992; Tosetti, 1998). In fact, TEA inhibits Kv1.3 channels underlying the delayed rectifier channels in SH-SY5Y cells (Friederich, 2003). When tested in the present study in the presence of the HERG inhibitor pimozide, the K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux was reduced by 100 % compared to TEA by itself which reduced the efflux by 30 %. Hence, TEA was selected in the present study to inhibit non HERG K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux in determining drugs effect on the HERG K\textsuperscript{+} channel in SH-SY5Y cells.

The effect of known HERG inhibitors on K\textsuperscript{+}-induced Rb\textsuperscript{+} efflux was determined in the presence of TEA to test the possibility of using the SH-SY5Y as a screening system. Drugs of various classes that are known to have an effect on HERG were tested including antihistamines, antidepressants, neuroleptic and antiarrhythmics
Pimozide, a neuroleptic drug reduced K+-induced Rb+ efflux in the present study and resulted in an IC50 of 37.8 nM. This value is 2-fold higher than that reported for pimozide inhibition of HERG in HEK293 cells using the patch clamp technique (Kang, 2000; Kongsamut, 2002). Compared to the Rb+ assay tested in HERG transfected HEK293 cells, the IC50 value of pimozide was almost the same. However it was 130-fold lower than that obtained using a fluorescent assay for HERG transfected HEK293 cells (Dorn, 2005).

The antihistamine, terfenadine was tested in various concentrations to determine the half maximal inhibition (IC50) value in SH-SY5Y cells. Terfenadine (30 µM, the maximum concentration tested) caused a maximal inhibition of 80 % of the K+-induced Rb+ efflux. These results are broadly in accordance with those reported by Taglialatela (1998) for terfenadine, where it inhibited 80 % of the current at a concentration of 3 µM in SH-SY5Y cells using the patch clamp technique. The IC50 of the antihistamine was also compared to data obtained in HERG transfected HEK293 from the literature which gave almost exact data (Crumb, 2000; Wang, 2003).

Amitriptyline, a psychotic drug, reduced K+-induced Rb+ efflux in the present study and resulted in an IC50 of 13 µM. This value is less than 1.5-fold higher than that reported for amitriptyline inhibition of HERG in HEK293 cells (Table 3-1) using the patch clamp technique (Tie, 2000b). Compared to the Rb+ assay tested in HERG transfected HEK293 cells, the IC50 value of amitriptyline was almost the same. This further indicates that the Rb+ assay may result in reliable estimates of drug potency depending on the system tested.
Spironolactone slightly reduced K⁺-induced Rb⁺ efflux in the present study; however no IC₅₀ value could be obtained. This drug is considered a weak inhibitor of HERG as it has an IC₅₀ value of 23 µM (Caballero, 2003). Interestingly, diltiazem another weak inhibitor that showed no activity in HERG transfected HEK293 (see section 2.4.6.4.11), was active in the SH-SY5Y cell line. It also resulted in an IC₅₀ value (15.6 µM) comparable to that in the literature (17.3 µM) and still provided weak inhibition (Zhang, 1999). This was expected as most drugs tested resulted in an IC₅₀ value 1.0-to 6-fold higher than those obtained using the patch clamp technique with quinidine sulfate being an exception causing 11-fold increase in IC₅₀ value. Hence, the IC₅₀ value for spironolactone in SH-SY5Y would have been well above the maximum tested concentration of 30 µM as it is 23 µM using patch clamped HERG transfected HEK293 cells (Caballero, 2003).

Two forms of quinidine were tested in this study as per Chapter 2, the commonly used quinidine sulfate and quinidine anhydrous. The reported IC₅₀ value for quinidine sulfate using patch clamping is 400 nM (Paul, 2002), which is 11-fold lower than that obtained in this study. Results of quinidine’s effect on K⁺-induced Rb⁺ efflux in the current study in SH-SY5Y were of similar potency ranking as in HERG transfected HEK293 cells, where quinidine sulfate was more potent than quinidine anhydrous.

This current study reports the use of the SH-SY5Y cell line for developing a non-radioactive Rb⁺ assay for HERG testing. The cell line was used to determine the IC₅₀ values for 10 known HERG inhibitors and the results were correlated with those
obtained in patch clamped HERG transfected HEK293 cells from the literature and in the Rb+ assay in HERG transfected HEK293 (Chapter 2). No correlation was possible between studies carried in SH-SY5Y using the patch clamp technique and the Rb+ assay as no systemic patch clamp studies to date have determined the IC50 values of HERG inhibitors in the SH-SY5Y cell line, however drugs such as terfenadine and astemizole were tested at one concentration (Taglialatela, 2000; Taglialatela, 1998). The data obtained using the Rb+ assay showed excellent correlation with those obtained in the literature using the patch clamped HERG transfected HEK293 cells and the Rb+ assay. The data obtained were of underestimated potency but compared to patch clamp resulted with a correlation coefficient of 0.82. The correlation coefficient obtained when compared to HERG transfected HEK293 cells using Rb+ assay was 0.92. The correlation study prove the suitability of using a constitutive HERG cell line (SH-SY5Y) for drug testing over transfected cells using the Rb+ assay, as it showed better correlation with patch clamped cells compared to the transfected cells (see Chapter 2).

Using the Rb+ assay for SH-SY5Y testing, 80 % of the tested compounds had an IC50 below 30 µM, the maximum concentration tested. Spironolactone and desipramine are weak inhibitors of HERG that did not result in IC50 values as it was expected that the value would be above the maximum concentration used in the assay. The percentage of active drugs was higher than that obtained in CHO cells using a fluorescent potentiometric assay (Dorn, 2005) and the Rb+ assay tested in HERG transfected HEK293 cells (Chapter 2). This study provides evidence that native HERG cell lines are suitable for screening assays using the functional Rb+ assay. The
suitability of the reliability of the assay over transfected cells could be due a critical role for HERG contributing to setting the membrane potential of the cells. It has been reported that some excitable mammalian cell lines express splice variants of HERG such as human tumor cells, human heart, rat tissue and mouse cardiac cells (Bianchi, 1998; Kirchberger, 2006; Kupershmidt, 1998; London, 1997). The human SH-SY5Y also expresses HERG splice variants known as HERG1 and HERG1B (Crociani, 2003) that could be involved in clamping the membrane voltage at depolarized values due to the lack of classical inward rectifier channels (Arcangeli, 1995a). This could explain the differences obtained in drug response in the SH-SY5Y and the HERG transfected HEK293 cells to depolarization by high K⁺ solution. The native HEK293 cells do not express these HERG splice variants that might be required to contribute to the control of the membrane voltage.

A [³H]-dofetilide binding assay has also been used as an indicator of HERG inhibition (Finlayson, 2001b). However, the native HEK293 and CHO cell lines which do not contain HERG have similar binding affinity to dofetilide as the SH-SY5Y cell line making the assay unsuitable for drug testing. Another study on the uses of the [³H]-dofetilide binding assay in HERG transfected HEK293 cells has shown good correlation to results obtained in patch clamped HERG-transfected HEK293 cells (Diaz, 2004). The study did not include testing on native HEK293 cells that do not express the HERG protein. The results of the reported study should be reassessed as Finlayson (2001b) reported that potent HERG inhibitors such as E-4031 had the same affinity in native HEK293 (not transfected transfected) as in SH-SY5Y cells. The good correlation obtained using the [³H]-dofetilide binding assay to patch clamp results
could be due to drug binding to a protein expressed in the HEK293 cells other than HERG. Thus, the human neuroblastoma cell line SH-SY5Y may be a suitable testing system for HERG inhibition. Both the Rb⁺ assay in the current study and in [³H]-dofetilide binding assay (Finlayson, 2001b) show its possible use and reliability in determining HERG inhibition compared to transfected cells which may contribute in false estimations of HERG inhibition.

Another drug binding assay has been developed to determine drug inhibition of HERG using the HERG transfected HEK293 cell line. The [³H]-astemizole binding assay uses the potent HERG inhibitor, astemizole to determine other drugs potency by competing with the same site as astemizole in the cell line (Chiu, 2004). The assay may be used to predict the rank order of the binding affinity of drugs. The correlation between the obtained binding affinity values and those obtained using patch clamping in HERG transfected HEK293 cells produced a coefficient of 0.91. This technique produces reliable results compared to the [³H]-dofetilide binding assay (discussed above) as astemizole has shown no significant binding to wild type HEK293 cells. The radioligand assay cannot be compared to the system used in this study as the cell lines are different and only 2 out of the 14 drugs used in the binding assay were used in our study.

An Rb⁺ assay using HERG transfected HEK293 cells resulted in a correlation coefficient when compared to patch clamping in the same cell line of 0.83 (Rezazadeh, 2004). The correlation study resulted in a similar coefficient to that obtained in the current study. This provides evidence that the assay developed using
SH-SY5Y cells is reliable in predicting drug toxicity and ranking potency. However, it needs to noted that the study reported by Rezazadeh et al, used a slightly different incubation conditions to those used in the current study which would explain the superiority of using the transfected HEK293 cells. The author incubated test drugs in open channel buffer which contains high K⁺ that is also used for channel activation. This buffer allows drugs better access to the HERG pore compared to the wash buffer used in the current study. Also only 3 out of the 15 tested drugs were used in the current study.

In conclusion, this study provides evidence for the suitability of the use of constitutive HERG cell lines for safety screening. The use of pharmacological interventions was required to eliminate non HERG K⁺-induced Rb⁺ efflux. Results of the study were in excellent correlation to those obtained using patch clamped HERG transfected HEK293 cells and using the Rb⁺ assay.
Chapter 4 Inhibition of the HERG K⁺ channel by the antidepressant clomipramine

4.1 Summary:

1. The effect of the antidepressant clomipramine on HERG function was tested. Three assay systems were used to determine the effect of clomipramine on HERG encoded K⁺ channels.

2. Using whole cell patch clamp electrophysiology, HERG currents in HERG transfected HEK293 cells were recorded in the presence of clomipramine in the external solution. Clomipramine inhibited HERG currents with an IC₅₀ value of 0.50 ± 0.32 µM.

3. Using a Rb⁺ efflux assay, clomipramine inhibition of HERG K⁺ channels was tested in HERG transfected HEK293 cells and in native HERG K⁺ channels in the neuroblastoma cell line SH-SY5Y. The IC₅₀ value obtained was 8.35 ± 0.03 µM and 2.18 ± 0.05 µM respectively.

4. Results of this study suggest clomipramine’s effect on the change in the QT interval may be caused by the inhibition of the HERG K⁺ channel.
4.2 Introduction

4.2.1 Effect of anti-depressants on the HERG K+ channel activity

Many non-cardiovascular drugs are capable of causing arrhythmias and torsade de pointes in human. These drugs include psychotropic drugs such as neuroleptic, antipsychotics, antidepressants and antianxiety drugs (Witchel, 2003). Arrhythmias caused by changes in the duration of the QT interval have been reported for the antidepressants amitriptyline (Davison, 1985; Dorsey, 2000; Manikoth, 1999), clomipramine (Flugelman, 1982), doxepin (Alter, 2001; Strasberg, 1982b), maprotiline (Curtis, 1984; Herrmann, 1983; Rialan, 1996), fluoxetine (McAnally, 1992) and cyclobenzaprine (Michalets, 1998). The main effect of these drugs have on cardiac tissues is a prolongation in the QT interval (Vieweg, 2004b) and not other ECG changes. These results indicate that tricyclic antidepressants have an effect on a component critical in the regulation of the QT interval, most likely to be the delayed rectifier channels encoded by HERG as has been reported for amitriptyline (Jo, 2000). Changes in the QT interval by amitriptyline, fluoxetine and maprotiline have been associated with severe arrhythmias known as polymorphic ventricular tachycardia and they have been shown to inhibit the HERG K+ channel in Xenopus oocytes (Ferrer-Villada, 2006; Jo, 2000; Thomas, 2002a).

Clomipramine is a tricyclic antidepressant used in patients with depression and obsessive behaviour (Deveaugh-Geiss, 1992). It is a derivative of the tricyclic antidepressant imipramine and has similar properties to other tricyclic antidepressants inhibiting norepinephrine and serotonin uptake into central nerve terminals (Gur, 1999; Ross, 1983b). Clomipramine has been proposed to have
quinidine-like effect on cardiac cells and has been reported to cause a prolongation in the QT interval in some patients. The quinidine-like effect is described as alterations in cardiac conduction and elevation of heart rate (Glassman, 1981). However no studies to date have discussed the mechanism of prolongation of the QT interval by clomipramine at the cellular level. Clomipramine may cause marked prolongation in the QT interval particularly when administrated with the antipsychotic drug risperidone (Sala, 2005). Short term clomipramine administration in healthy dogs causes a decrease in the heart rate, however no potentially dangerous arrhythmias or tachycardia have been detected (Pouchelon, 2000). Intravenous injections of clomipramine in conscious rats causes a decrease in heart rate and prolongation in the QT interval (Forsberg, 1983). A decrease in cardiac function has also been detected in isolated rabbit hearts upon clomipramine treatment (Nielsen-Kudsk, 1980).

In this study the electrophysiological properties of HERG channels in the established HERG transfected HEK293 cell line were studied at 37°C. The cell line was also used to investigate whether clomipramine inhibits the HERG K⁺ channel in HERG transfected HEK293 cells. The aim was determine whether clomipramine’s inhibition of the HERG K⁺ channel could be the explanation for cardiovascular events.
4.3 Methods

4.3.1 Patch Clamp

4.3.1.1 Cell preparation
As per section 2.3.5.1

4.3.1.2 Preparation of patch pipettes
As per section 2.3.5.2

4.3.1.3 Whole cell recording
As per section 2.3.5.3

4.3.2 Software configuration
As per section 2.3.8

4.3.3 Solutions
External solution contained (mM): 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES; pH 7.4 with NaOH. The osmolarity of the external solution was approximately >10% higher than the osmolarity of the internal solution to avoid cell burst which may occur due to this difference. This was checked by using an osmometer. The osmolarity of the solutions used in this study was adjusted by the addition of sucrose to the external solution. Clomipramine was added to the external bathing solution.
The electrode internal solution contained (mM): 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES; pH 7.2 with KOH.

4.3.4 Rubidium efflux assay

As per section 2.3.6.1.2.

4.3.4.1 Rubidium determination using atomic absorption spectroscopy

As per section 2.3.6.1.3.

4.3.4.2 Statistics and calculations

All data were expressed as the mean and standard error of the mean (SEM) with the number of observations (n) being given. Data manipulation, IC₅₀ calculations and graphical presentations were completed in GraphPad Prism 4 software package (GraphPad software, Inc, San Diego, CA).

Activation curves were fitted using a Boltzmann equation to determine the half maximum activation voltage as follows:

\[ I = \frac{I_{\text{max}}}{1 + \exp \left( \frac{V_{1/2} - V_m}{k} \right)} \]

Where I is the tail HERG current amplitude at the test potential \( V_m \), \( I_{\text{max}} \) is the maximal HERG tail current observed, \( V_{1/2} \) is the potential at which HERG current is half maximally activated, k is the slope factor describing HERG current activation.
Dose-response curves were fitted by a standard Hill equation to calculate the IC₅₀ value as follows:

\[
\text{Fractional block} = \frac{1}{1 + (\text{IC}_{50}/[\text{drug}])^{n_{\text{H}}}}
\]

Where IC₅₀ is the concentration at which half the maximal inhibition of HERG currents occurs and nₜ is the Hill coefficient for the fit.

To determine the fractional block of current at each voltage, the following equation was applied:

\[
\text{Fractional block} = 1 - \left( \frac{I_{\text{HERG} + \text{drug}}}{I_{\text{HERG}}} \right)
\]

Where \( I_{\text{HERG} + \text{drug}} \) are the HERG tail current amplitude in the presence of drug and \( I_{\text{HERG}} \) is tail amplitude in control cells.
4.4 Results

4.4.1 Concentration-dependent I\(_{\text{HERG}}\) inhibition by clomipramine

To study the effect of clomipramine on HERG currents in HERG transfected HEK293 cells, an activation protocol was applied as in Figure 4-1. From a holding potential of -80 mV, I\(_{\text{HERG}}\) was activated using a 10 mV step for 2 s to +20 mV and tail currents were induced by a single 4 s repolarising step to -40 mV. The protocol was applied repeatedly up to 100 sweeps to monitor changes prior to and after clomipramine addition to the external bathing solution. Peak HERG tail current amplitude measured using the repolarising step to -40 mV was taken as a measure of the degree of current inhibition by clomipramine. HERG tail currents (I\(_{\text{HERG}}\)) were inhibited after a 6 min exposure to 1 µM clomipramine (Figure 4-1). This inhibition is evident during the activation states of the channel as tail currents are a measure of channel activation. As seen in Figure 4-1, this inhibition was partially reversed after wash out of the drug.

Figure 4-2 and Figure 4-3, shows the time-dependence of I\(_{\text{HERG}}\) inhibition by clomipramine for the same currents recorded in the same cell as in Figure 4-1. The maximal inhibition caused by clomipramine occurred at 6 min. To determine the half maximum dose for I\(_{\text{HERG}}\) inhibition, four concentrations between 10 nM and 10 µM of clomipramine were tested and a concentration–response curve was constructed (Figure 4-4). The IC\(_{50}\) value for I\(_{\text{HERG}}\) by clomipramine derived from the fitted curve was 500.19 ± 0.32 nM.
Fig 4-1

A

Control

B

1μM Clomipramine

C

Wash-off

2000 ms
Figure 4-1: Effect of clomipramine on HERG currents in HERG transfected HEK293 cells.

Panel A: representative currents in the absence of clomipramine elicited by the protocol below the trace. Panel B: representative currents in the presence of clomipramine (1 µM) for 10 min elicited by the protocol below the trace in the same cell in panel A. Panel C: representative currents following wash-off of clomipramine (1 µM) for 5 min elicited by the protocol below the trace in the same cell in panel A and B.
Figure 4-2: Time-dependent effect of clomipramine (1 μM) on $I_{\text{HERG}}$.

Representative currents, from the cell in Figure 4-1, in the absence and following clomipramine (CMI) addition, elicited by the voltage-protocol shown. Coloured traces represent HERG currents recorded at different times after start of exposure to clomipramine (1 μM).
Figure 4-3: Time-dependent effect of clomipramine (1μM) on tail currents.

Each sweep was 6000 ms long as per protocol in Figure 4-1. The mean recordings of $I_{HERG}$ tail at each time point was normalized to the maximum current prior to clomipramine addition. The plot represents the degree of block over time by clomipramine. An increase in fractional block was observed over time with a maximum block occurring at 2 min. Data represents mean recordings ± SEM (n = 3).
Figure 4-4: Concentration-response curve for clomipramine tested in HERG transfected HEK293 cells.

Four clomipramine concentrations were tested. The plot represents tail currents normalized to maximum current obtained in the absence of clomipramine. Data points were fitted with a standard Hill equation, which yielded an IC$_{50}$ value of 500.19 ± 0.32 nM. The plotted curve had a fit coefficient ($r^2$) of 0.80. Each drug concentration was added to 3 - 8 cells. * Denotes significantly different from currents obtained prior to clomipramine addition ($P < 0.05$, Student’s t-test).
4.4.2 Voltage-dependence of clomipramine inhibition

In order to determine whether $I_{HERG}$ inhibition by clomipramine was voltage-dependent, 4 s depolarisations were applied to a range of voltages between -60 mV and +50 mV to activate $I_{HERG}$. The cell was then clamped to -50 mV for 5 s to record tail currents. A recording of the current was taken prior to clomipramine addition for 5 min, during and after applying external buffer as a wash-off step. Figure 4-5 displays an example of $I_{HERG}$ inhibition by clomipramine over all test voltages after 5 min. Clomipramine significantly attenuated HERG currents at voltages above 0 mV (Figure 4-6) and had no effect in the range -60 to -20 mV when data was normalized against maximum in control. The clomipramine effect was reversed by a 5 min wash-off step. The tail currents were also normalized against the maximum current obtained in the absence of clomipramine in the same cell to determine the change in the size of the tail current in the presence of clomipramine. Clomipramine caused a significant leftward shift (-28.03 ± 0.60 mV) in the half-activation voltage for HERG compared to control (-21.61 ± 0.95 mV) (Figure 4-6, Figure 4-7). As illustrated in Figure 4-6, there was a significant decrease in the maximum current during the application of clomipramine. Clomipramine had an effect on the current-voltage relationship of HERG at voltages between -10 and 10 mV, where the maximum current occurs (Figure 4-8).
Fig 4-5
Figure 4-5: Voltage dependence inhibition of I\textsubscript{HERG} by clomipramine.

Panel A: Trace of a single cell prior to exposure to clomipramine. Panel B: Trace of the same cell in panel A following 6 min exposure to clomipramine (1 \textmu M). Panel C: Trace of the same cell in panels A and B following wash-off (5 min) from clomipramine (1 \textmu M). All currents were elicited by the voltage-protocol showing below each trace.
Figure 4-6: Effect of clomipramine on HERG tail currents (normalized against maximum in control)

Mean activation curves for HERG tail currents in the absence ($V_{1/2} = -21.61$ mV), and in the presence ($V_{1/2} = -28.03$ mV) of clomipramine (1 µM). Currents were normalized to maximum current in control. Data represents mean tail currents at each membrane voltage ($n = 4$) ± SEM. * Denotes significantly different from clomipramine ($P < 0.05$, ANOVA, followed by Bonferroni post test).
Figure 4-7: Effect of clomipramine on HERG tail currents (normalised against maximum in each treatment)

Mean activation curves for HERG tail currents in the absence ($V_{1/2} = -21.61$ mV), and in the presence ($V_{1/2} = -28.44$ mV) of clomipramine (1 µM). Currents were normalized to maximum current in each treatment. Data represents mean tail currents at each membrane voltage ($n = 4$) ± SEM. * Denotes significantly different from control ($P < 0.05$, Student t-test).
Figure 4-8: The effect of clomipramine on the current-voltage relationship of HERG currents.

The figure shows currents prior and after exposure to clomipramine (1 µM). The effect of clomipramine on the currents was also measured when a wash-off step was introduced by applying external buffer only. Data represents mean currents at each voltage (n = 4). * Denotes significantly different from clomipramine (P < 0.05, ANOVA, followed by Bonferroni post test).
4.4.3 Determination of HERG inhibition by Clomipramine using the Rb⁺ assay

The Rb⁺ assay developed in earlier chapters was employed. Using the test conditions outlined in chapter 2 in HERG transfected HEK293, a range of clomipramine concentrations between 1 nM to 30 µM were tested and a concentration-response curve was constructed in Figure 4-9. The IC₅₀ value obtained from the fitted graph was 8.35 ± 0.03 µM.

Clomipramine was also tested in the SH-SY5Y neuroblastoma cell line as per conditions outlined in chapter 3. The drug was tested over concentrations between 1 nM to 30 µM. The fitted dose-response (Figure 4-10) curve resulted in an IC₅₀ value of 2.24 ± 0.17 µM.

The differences between the IC₅₀ values calculated in this section and in section 4.4.1 using patch clamping were compared. The IC₅₀ value obtained from the Rb⁺ assay in HERG transfected HEK293 was significantly different from that obtained using the Rb⁺ assay in SH-SY5Y cells and patch clamped HERG transfected HEK293 cells (P < 0.05, ANOVA, followed by Bonferroni’s test) (Table 4-1). The IC₅₀ value obtained from the Rb⁺ assay in SH-SY5Y cells was also significantly different from the patch clamped HERG transfected HEK293 (P < 0.05, ANOVA, followed by Bonferroni’s test) (Table 4-1).
Figure 4-9: Effect of clomipramine on K⁺-induced Rb⁺ efflux in HERG-transfected HEK293 cells.

The Rb⁺ efflux was expressed as a ratio of each efflux value to the maximum efflux in the absence of clomipramine. K⁺-evoked Rb⁺ efflux in the presence of 10 clomipramine concentrations were used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 8.35 ± 0.03 µM. Data represents mean ± SEM (n = 6). The curve was generated by a non-linear curve fitting. The goodness of the fit (r²) was 0.96. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Figure 4-10: Effect of clomipramine on K⁺-induced Rb⁺ efflux in neuroblastoma (SH-SY5Y) cells.

The Rb⁺ efflux was expressed as a ratio of each efflux value to the maximum efflux in the absence of clomipramine. K⁺-evoked Rb⁺ efflux in the presence of 10 clomipramine concentrations were used to construct a concentration-response curve. The IC₅₀ value obtained from the curve was 2.24 ± 0.17 μM. Data represents mean ± SEM (n = 6-10). The curve was generated by a non-linear fitting. The goodness of the fit (r²) was 0.622. * Denotes significantly different from 1 (P < 0.05, ANOVA, followed by Dunnett’s test).
Table 4-1: Differences between IC\(_{50}\) values obtained in the three methods

<table>
<thead>
<tr>
<th>Method and system</th>
<th>IC(_{50}) (µM)</th>
<th>Significant from Rb(^+) assay using HERG transfected HEK293 cells?</th>
<th>Significant from Rb(^+) assay using SH-SY5Y cells?</th>
<th>Significant from Patch clamping using HERG transfected HEK293 cells?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb(^+) assay using HERG transfected HEK293 cells</td>
<td>8.35 ± 0.03</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
</tr>
<tr>
<td>Rb(^+) assay using SH-SY5Y cells</td>
<td>2.24 ± 0.17</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
<td></td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
</tr>
<tr>
<td>Patch clamped HERG transfected HEK293 cells</td>
<td>0.500 ± 0.003</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
<td>Yes (P &lt; 0.05, ANOVA, followed by Bonferroni’s test).</td>
<td></td>
</tr>
</tbody>
</table>
4.5 Discussion

4.5.1 Inhibition of HERG currents by clomipramine

Clomipramine is an antidepressant that has been reported to cause prolongation of the QT interval in humans (Leonard, 1995), however its mechanism of action on the heart that leads to such cardiotoxic effects has not been investigated previously. Changes in the QT interval by drug intake has been linked to the inhibition of the HERG K\(^+\) channel underlying the delayed rectifier current responsible for the repolarization phase in ventricular myocytes (see section 1.4). This study provides evidence that HERG channels are inhibited by clomipramine with an IC\(_{50}\) of 8.35 µM in HERG transfected HEK293 and 2.24 µM in SH-SY5Y using the Rb\(^+\) assay; and using the voltage clamp technique resulted in an IC\(_{50}\) of 0.5 µM in HERG transfected HEK293.

In the clinical situation there is a wide range of plasma concentrations of clomipramine due to differences in individual metabolism, particularly through the cytochrome P450 system and in particular P450 2D6 (Linder, 1998). Reports vary with plasma levels as high as 3 µM (Szegedi, 1996) with a target range of 1 µM (Balant-Gorgia, 1991; Charlier, 2000) and levels as low as 0.1 µM being reported. Clearly the concentrations of clomipramine in the present study are in the target range. In the present patch clamp study, 1 µM clomipramine had a marked effect on the tail currents with a block approaching 80 %. Using the Rb\(^+\) assay in HEK 293 cells, 1 µM was at the threshold for the inhibitory effect. These values are in agreement with reports of ECG changes found in children treated with clomipramine.
and had a drug plasma level in the range 0.349 - 3 μM (Leonard, 1995). This suggests a link between HERG inhibition by clomipramine and prolongation in the QT interval.

This present study was also designed to investigate clomipramine’s mechanism of inhibition on the HERG K⁺ channel using the voltage clamp technique. Clomipramine inhibition of the HERG K⁺ channel in HERG transfected HEK293 was detected using the voltage clamp technique. Blockade of HERG K⁺ currents displayed voltage- and concentration-dependence. To determine the IC₅₀ value for HERG inhibition by clomipramine a single voltage protocol was used holding at -80 mV and stepping to 0 mV where the maximum current occurs. Onset of current inhibition was rapid reaching a maximum at 2 min but was also reversed upon washout. This rapid onset of block has been reported for HERG inhibitors such as the anti-arrhythmic drug ajmaline, the antidepressant fluoxetine and for several antimalarial agents such as halofantrine, chloroquine and mefloquine (Kiesecker, 2004; Thomas, 2002a; Traebert, 2004). This reversal effect of the drugs is common and is likely related to the lipophilicity of the drugs and their partitioning in the membranes.

HERG inhibition by clomipramine also displayed voltage-dependence when a protocol with several voltage steps was employed. Inhibition of the channel occurred at voltages positive to 0 mV, consistent with previously reported HERG inhibitors such as halofantrine (Mbai, 2002). These results indicate that clomipramine acts on a binding site in the channels pore and requires the channel to be in its fully active state to act on it. Since tail currents may represent full channel activation, a reduction
in the tail current could indicate inhibition of the open state of the channel at maximal currents. A shift in the half maximum voltage towards more negative voltages was also detected, indicating its effect on the voltage-dependency of the channel. This effect has been reported for the antimalarial drug halofantrine, which also displays voltage-dependency of inhibition (Tie, 2000a). Voltage-dependence of channel inhibition is a property of open-state inhibitors of HERG such in dofetilide (Snyders, 1996a). The negative shift in $V_{1/2}$ has been reported for the antidepressant maprotiline (Ferrer-Villada, 2006) and the antihistamine clemastine (Ridley, 2006). Clomipramine has been reported to possess quinidine-like cardiac effects, similar to those reported for maprotiline (Burckhardt, 1978) which is also a HERG inhibitor (Ferrer-Villada, 2006). The observed mechanism of HERG inhibition by clomipramine in a voltage-dependence manner is also similar to antidepressants such as amitriptyline (Jo, 2000). It has been reported that most HERG K⁺ channel blockers act on either the active or inactive states of the channel (Ficker, 1998) as the drug binding site has been proposed to be located in the channel’s inner pore (Sanguinetti, 2005).

### 4.5.2 HERG inhibition in relation to clinical observations

Many tricyclic antidepressants have an inhibitory effect on the HERG K⁺ channel (Jo, 2000; Thomas, 2002b) leading to QT prolongation and Torsade de Pointes including amitriptyline, imipramine, haloperidol, thioridazine and sertindole. Clomipramine induces ECG changes including tachycardia in children and adults suffering from obsessive-compulsive disorder (Leonard, 1995). Clomipramine shows very similar chemical properties to other tricyclic antidepressants such as amitriptyline and
maprotiline. It has been postulated that inhibition of HERG by drugs requires π-stacking of aromatic rings on the drugs in the channel’s binding site (Lees-Miller, 2000; Mitcheson, 2000a). Clomipramine, maprotiline and amitriptyline have similar arrangements of aromatic rings in their structures allowing this to occur (Figure 4-11).

4.5.3 Choice of testing method

ECG parameters in animals such in dogs (Reich, 2000) and rats (Forsberg, 1983) has provided conflicting results for clomipramine compared to those obtained in humans. Testing of clomipramine in healthy dogs with behavioural conditions for a month resulted in no change in ECG parameters, whilst clomipramine administration in humans leads to a prolongation in the QT interval (Glassman, 1981). Therefore, testing clomipramine in in vitro assays using human channels might provide a more predictive tool.

Testing the effect of drugs on the HERG K+ channel is common in cultured cell lines; however regulatory bodies have not set a recommended testing technique or system.

In this present study three different approaches for clomipramine testing on the HERG K+ channel were employed including the Rb+ assay using a cell line natively expressing the HERG K+ channel (SH-SY5Y, see chapter 3) and a HERG transfected HEK293 (see chapter 2); the patch clamp technique is the gold-standard technique for testing (see section 2.2.3.4). Though there was a 3-fold difference in the IC50 in SH-SY5Y (Rb+ assay) compared to the patch clamp method, the results closer than the IC50 obtained using the transfected cells (Rb+ assay) which resulted in an IC50 17-fold
greater than that obtained using patch clamp. Both the patch clamp and the Rb⁺ assay have advantages and disadvantages. The patch clamp is considered the most reliable, as it provides changes in current amplitude and changes in the parameters most affected by the drug. However, this method is labour intensive and time consuming. The Rb⁺ assay does not provide information on how the drug can affect the activation and the inactivation states of the channel or the voltage dependency of block, however results of the SH-SY5Y Rb⁺ assay show that more accurate IC₅₀ values can be generated and are comparable to those obtained in patch clamp. In fact most drugs had IC₅₀ values that were almost identical to those obtained using the patch clamp technique. These results confirm the validity of the SH-SY5Y cell line for drug testing using the Rb⁺ assay as discussed in chapter 3. The results indicate that the Rb⁺ assay is suitable for drug safety testing; however the assay sensitivity is dependent on the cell line used. As mentioned previously in the discussion, the question of in vivo relevance is also important. It may be that the greater sensitivity of patch clamp electrophysiology actually overestimates HERG inhibition. This is important as therapeutically useful drugs may be screened out even though their therapeutic concentrations may not affect HERG.
The chemical structure of the antidepressants: amitriptyline, maprotiline and clomipramine. The three anti-depressants are very similar in structure containing 3 aromatic rings at one end. This aromatic structure may explain their inhibitory effect on the HERG K⁺ channel.
Chapter 5 PKC regulation of HERG currents in HEK293 cells

5.1 Summary

1. Effect of activation of protein kinase C on HERG K⁺ channels was examined in HERG transfected HEK 293 cells using patch clamp electrophysiology.

2. Phorbol 12, 13-diacetate (PDA), a PKC activator was tested at two concentrations to determine its effect on HERG currents. PDA (1 μM) induced a decrease in HERG tail currents. This reduction in outward current was time-dependent as it increased over time. PDA (3 μM) was also tested over 3 min. This concentration caused a larger reduction in tail currents.

3. To determine whether the decrease was due to a specific effect on PKC, the selective PKC peptide PKC [19-36], was included in the patch pipette solution. PKC [19-36] (1 μM) applied intracellularly, did not cause a change in the response to PDA (1 μM). Increasing the concentration of PKC [19-36] to 20 μM had no significant effect on HERG currents in the presence of 1 μM PDA. PKC [19-36] (20 μM) had no effect on HERG currents when applied intracellularly in the absence of PDA.

4. The results of the study indicate that PKC does not regulate HERG in transfected HEK293 cells and that PDA might have direct inhibition of the channel or may be activating other proteins that regulate HERG function.
5.2 Introduction

5.2.1 Protein Kinase C

Protein kinase C represents a family of enzymes that alter proteins by phosphorylation. Phosphorylation occurs by donating a phosphate group from adenosine triphosphate (ATP) to the targeted protein which alter its function. Phosphorylation of proteins arises via the covalent binding of the donated phosphate to the free hydroxyl group on amino acids such as serine and threonine (Asaoka, 1992; Hug, 1993). Initial studies suggested that activation of PKC is caused by Ca$^{2+}$ and phospholipids (Kikkawa, 1983; Nishizuka, 1984) but this has been modified as described below (Figure 5-1). PKC is also activated by diacylglycerol, a product of the hydrolysis of the membrane inositol trisphosphate. Diacylglycerol causes full activation of PKC in several systems even at basal levels of Ca$^{2+}$ (Murakami, 1986).

PKC plays a vital role in cellular processes such in proliferation (Kanashiro, 1998; Musashi, 2000), regulation of cardiac contractility (Bowling, 1999; Takeishi, 1998), apoptosis (Barr, 1997; Berra, 1997) and the regulation of ion channels (Cole, 1996; Kamp, 2000; Schreibmayer, 1999; Swope, 1999; Walsh, 1988).

In total, there are 11 PKC isoforms subdivided into 3 subfamilies; these are classified as either Ca$^{2+}$ dependent or independent. The Ca$^{2+}$ dependent subfamily are also phospholipid and diacyl glycerol dependent and are known as the conventional PKC isoforms. The conventional PKC subfamily are α, β1, β2 and γ. This subfamily of PKC differs in structure to other isoforms by the presence of a region known as the C2 domain which binds anionic phospholipids in a Ca$^{2+}$ dependent manner (Steinberg,
Chapter 5

1995). The Ca$^{2+}$ independent PKC subfamily is known as novel PKC and comprises of δ, ε, η and θ. This subfamily has the same structure as the conventional except for the absence of the Ca$^{2+}$ modulation site (Pappa, 1998). It is also activated by phospholipids and diacyl glycerol. The third PKC subfamily is the atypical and this one differs to the conventional and novel where it cannot be activated by diacyl glycerol or Ca$^{2+}$. This subfamily includes the isoforms ξ, ι and λ and are activated by phosphatidyl serine, phosphatidyl inositol trisphosphate and the octapeptide angiotensin II (Liao, 1997; Nakanishi, 1993).

5.2.2 Phosphorylation of HERG

The HERG encoded K$^{+}$ channels are phosphorylated by several protein kinases including the serine/threonine protein kinases protein kinase A and protein kinase B in HERG transfected HEK293 cells (Thomas, 1999; Zhang, 2003). The rat homologue of HERG known as r-erg1 has been shown to be phosphorylated by Src tyrosine kinase in the MLS-9 cell line (Cayabyab, 2002).

The HERG protein contains phosphorylation sites for tyrosine phosphorylation as well as sequence motifs for cystolic protein-tyrosine kinase (PTKs) (Cayabyab, 2002). Basal function of the HERG K$^{+}$ channel homologue in rat requires regulation by the tyrosine kinase Src tyrosine kinase in MLS-9 cells. Reduction in the basal levels of Src causes reduction in r-erg1 K$^{+}$ currents. An elevation of the Src peptide levels shifts the channels activation towards more negative voltages (Cayabyab, 2002). This tyrosine kinase affects erg K$^{+}$ currents in an opposite manner to serine/threonine protein kinases (see below).
The HERG protein contains four PKA consensus sites in its cytoplasmic terminals (Cui, 2000; Thomas, 1999). PKA phosphorylates the HERG protein by directly acting on its phosphorylation sites in a cAMP-dependent manner (Cui, 2000) (Figure 5-1).

The PKA phosphorylation process has been linked to 14-3-3 proteins, which bind to regions on targeted proteins and alter their function and conformation (Ichimura, 1997; Xiao, 1995). Two isoforms of 14-3-3 proteins, the η and ε bind to the HERG protein in vitro (Kagan, 2002). The protein plays a role in enhancing cAMP and PKA effects on the HERG K+ channel by binding to specific sites the N- and C-terminal of the channel (Choe, 2006; Kagan, 2005; Tutor, 2006). HERG phosphorylation by PKA reduces HERG tail currents in HEK293 cells. PKA phosphorylation of the HERG K+ channel results in alterations in channel function. Changes in function include reduction in current, increase in deactivation and voltage-dependent activation shifts to more positive voltages (Cui, 2000; Kiehen, 1998; Wei, 2002). Interestingly, binding of 14-3-3 to HERG K+ channels reduces HERG currents (Choe, 2006; Kagan, 2002), which further suggests the proteins role in regulating PKA phosphorylation of the channel.

The HERG K+ channel is regulated by another protein kinase in the serine/threonine protein kinase family, known as protein kinase B (PKB). PKB enhances HERG K+ currents in HERG transfected HEK293 cells and a reduction in PKB basal levels significantly reduces HERG currents (Zhang, 2003).

HERG K+ channel modulation by α1-adrenoreceptors has been investigated in Xenopus oocytes. Phenylephrine, an α1-adrenoreceptor stimulant causes a shift in the
half maximum activation voltage of HERG K+ channel towards positive voltages (Thomas, 2004b). Prazosin, a competitive inhibitor of the receptor prevented the shift in the $V_{1/2}$ caused by phenylephrine, which confirms the role of the $\alpha_1$-adrenoreceptors in HERG K+ regulation. A role effect of PKC and PKA in this process was suppressed by stimulating the $\alpha_1$-adrenoreceptor in the presence of PKA and PKC specific inhibitors. The effect of the $\alpha_1$-adrenoreceptor stimulation was also still seen in HERG K+ channels lacking the PKA and PKC phosphorylation sites (Thomas, 2004b).

The contribution of PKC to the regulation of HERG K+ currents has been determined using phorbol esters in expression systems such as Xenopus oocytes (Thomas, 2003) and Chinese hamster ovaries cells (CHO). It has been reported that PKC regulates the HERG K+ channel by indirect phosphorylation of the channel by acting on HERG via intermediate proteins (Thomas, 2003). The addition of the PKC activator, phorbol-12-myristate-13-acetate (PMA) (100nM) caused a delay in the activation of the HERG K+ channel by a causing a shift in the activation voltage in which the $V_{1/2}$ shifted from 1.2mV to 38.7mV in Xenopus oocytes (Thomas, 2003). This effect of PKC was abolished by treatment with the specific PKC inhibitor Ro-32-0432 (3μM), however a small shift in the activation curve was still evident. Studies on mutant HERG K+ channels lacking the PKC phosphorylation site have still shown PMA’s ability to affect the channel which suggests that PMA acts independently of these phosphorylation sites (Thomas, 2004b; Thomas, 2003). The authors justified PMA’s effect on HERG as indirect of PKC phosphorylation of HERG and is mediated by other proteins.
5.2.3 Phorbol esters

In vivo, protein kinase C isoforms are activated by diacylglycerol and phospholipids that are hydrolysed from cells plasma membranes. In vitro, a class of potent tumour promoters known as phorbol esters activate novel and conventional PKC isoforms (Roivainen, 1993a; Roivainen, 1993b). Phorbol esters exert some of their actions on proteins other than protein kinase C which have similar regulatory domains to the kinase (Kazanietz, 2000a; Ron, 1999). These proteins include chimaerins and Ras-guananyl releasing protein, see review by (Kazanietz, 2000b). The phorbol esters used to activate PKC include phorbol-12, 13 myristate (PMA), phorbol 12, 13 dibutyrate (PDB) and phorbol 12, 13 diacetate (PDA).

To date, only phorbol-12, 13 myristate (PMA) and phorbol 12, 13 dibutyrate (PDB) have been tested to determine the effect of protein kinases on the HERG K⁺ channel and on the delayed rectifier channels (I_{Kr}) in Xenopus oocytes and guineapigs myocytes respectively. In the present study, phorbol 12, 13 diacetate (PDA) was tested in HERG transfected HEK293 cells to study the effect of PKC on HERG function using the patch clamp method. To determine whether the effect of PDA on HERG currents is linked to PKC activation, a specific PKC inhibitor was tested. The PKC [19-36] peptide is a specific PKC inhibitor peptide which acts on the pseudo substrate of the kinase. The pseudo substrate inactivates PKC by blocking its active site (House, 1987). Activation of the kinase occurs by the release of the pseudo domain. This inhibitor peptide was chosen to eliminate any possible direct interaction of specific PKC inhibitors on HERG as reported with bisindolylmaleimide I (Schledermann, 2001).
Figure 5-1: Proposed mechanism of HERG regulation by PKC and PKA. Unknown regulation mechanisms have been marked with a ? Based on data from Thomas (2004b) and Cui (2000).

PKC: protein kinase C  DAG: diacyl glycerol  IP3: 1, 4, 5-inositol-trisphosphate  PIP2: phosphatidyl-4, 5-bisphosphate
PLC: phospholipase Cβ  β-AR: beta adrenoreceptors  alpha-AR: alpha adrenoreceptors  AC: Adenyl cyclase  Gs and Gq: proteins
5.3  Methods

5.3.1  Patch clamp

Refer to section 4.3.4.

5.3.2  Solutions

All solutions were prepared as per section 4.3.3.

5.3.3  Drugs

4β-Phorbol 12, 13 diacetate (PDA) obtained from Alexis Cooperation (Lausen, Switzerland). Protein kinase C peptide inhibitor, PKC [19-36] was obtained from Calbiochem Biosciences (Darmstadt, Germany)

5.3.4  Drug preparation

Phorbol 12, 13 diacetate (PDA) was prepared in DMSO; aliquots were prepared and kept at -20 °C until used. The protein kinase C peptide inhibitor, PKC [19-36] was made up in internal solution. This was prepared to exclude further changes in internal osmolarity while adding the inhibitor peptide into the internal solution whilst recording. The peptide inhibitor was prepared as a stock at 500 µM and kept in aliquots at -20 °C until used.
5.4 Results

5.4.1 The effect of PDA on HERG K⁺ channel currents

A whole-cell patch clamp protocol was employed to determine the effect of the phorbol ester PDA, on HERG K⁺ currents in HERG transfected HEK293 cells (Figure 5-3). PDA was tested at two concentrations (1 µM and 3 µM) stepping from -80 mV to voltages between -60 and +50 mV and tail currents were recorded at -50 mV. Addition of PDA (1 µM) reduced HERG K⁺ tail currents in a time-dependent manner (Figure 5-2, Figure 5-3, Figure 5-4). The reduction in the tail currents was observed at voltages positive to -20 mV (Figure 5-4), the voltage at which the half maximum tail current is detected. In Figure 5-2 the reduction in tail currents was compared at 3 different voltages: -60, -10 and +50 mV. It can be observed from these voltages that a reduction in the tail current is detected at -10 and +50 mV; there were no tail currents detected at -60 mV. This effect on the tail currents is seen in the activation curve, where a significant reduction of tail current is observed between -20 and +50 mV (Figure 5-4). The consolidated traces of HERG activity between voltages of -60 and +50 mV show a reduction in the voltage-dependent activation of the channel (Figure 5-3). PDA caused a reduction in the overall size of currents over time (Figure 5-3). The effect of time on HERG K⁺ currents under control conditions showed no decrease in currents (Figure 5-6). PDA (1 µM) had no effect on the I-V relationship of HERG currents (Figure 5-5).

PDA (3 µM) was also tested in HERG transfected HEK293 cells (Figure 5-7). At this higher concentration, HERG tail currents were reduced significantly after 3 min
(Figure 5-8). Recordings and seals were lost after 3 min as the cells did not tolerate the high concentration of the phorbol ester.
Fig 5-2

A -60 mV

B -10 mV

C +50 mV
Figure 5-2: Effect of PDA (1 μM) on HERG K+ channel currents measured in a single HERG transfected HEK293 cells using the protocol outlined in Figure 5-3.

Each individual trace represents current measured at the applied voltage. **Panel A:**

The effect of PDA on HERG tail currents at -60 mV over 3 min intervals for 12 min.

**Panel B:** The effect of PDA on HERG tail currents at -10 mV over 3 min intervals for 12 min. **Panel C:** The effect of PDA on HERG tail currents at +50 mV over 3 min intervals for 12 min.
Figure 5-3: PDA-mediated effects on HERG K+ currents expressed in HERG transfected HEK293 cells.

Control cell prior to exposure to 1 µM PDA elicited by protocol outlined and post-exposure to PDA (1 µM) at 3, 6, 9 and 12 min. Cells were held at -80 mV and depolarised to voltages between -60 and +50 mV for 2 s, and then clamped to -50 mV for 4 s.
Effect of PDA (1 μM) on the activation curve of HERG in HERG transfected HEK293 cells

Fig 5-4

A

Normalized tail current

0 min

1 μM PDA 3 min

Membrane potential (mV)

0.2 0.4 0.6 0.8 1.0 1.2

0 -75 -50 -25 0 25 50 75

B

Normalized tail current

Control

1 μM PDA 6 min

Membrane potential (mV)

0.2 0.4 0.6 0.8 1.0 1.2

-75 -50 -25 0 25 50 75

C

Control

1 μM PDA 9 min

Normalized tail current

Membrane potential (mV)

0.2 0.4 0.6 0.8 1.0 1.2

-75 -50 -25 0 25 50 75

D

Control

1 μM PDA 12 min

Normalized tail current

Membrane potential (mV)

0.2 0.4 0.6 0.8 1.0 1.2

-75 -50 -25 0 25 50 75
Figure 5-4: PDA-mediated effects on the activation curve of HERG K⁺ currents expressed in HERG transfected HEK293 cells.

Cells were held at -80 mV and depolarised to voltages between -60 and +50 mV for 2 s, and then clamped to -50 mV for 4 s. **Panel A:** Control voltages compared to 3 min exposure to PDA (1 µM) elicited by protocol outlined in Figure 5-3. **Panel B:** Currents 6 min after exposure to PDA in the same cell as A. **Panel C:** Currents 9 min after exposure to PDA in the same cell as A. **Panel D:** Currents 12 min after exposure to PDA in the same cell as panel A. Data represents mean (n = 4) ± SEM. * Denotes significantly different from 0 min (P < 0.05, ANOVA, followed by Bonferroni post test).
Effect of PDA (1 μM) on the I-V relationship of HERG currents in HERG transfected HEK293 cells

A

B

C

D

Fig 5-5
**Figure 5-5: PDA-mediated effects on the I-V relationship of HERG K⁺ currents expressed in HERG transfected HEK293 cells.**

Cells were held at -80 mV and depolarised to voltages between -60 and +50 mV for 2 s, and then clamped to -50 mV for 4 s. **Panel A:** Control voltages compared to 3 min exposure PDA (1 µM) elicited by protocol outlined in **Figure 5-3.** **Panel B:** Currents 6 min after exposure to PDA in the same cell as A. **Panel C:** Currents 9 min after exposure to PDA in the same cell as A. **Panel D:** Currents 12 min after exposure to PDA in the same cell as panel A. Data represents mean (n = 4) ± SEM.
Fig 5-6

A

Normalized tail current

Membrane potential (mV)

B

Normalized tail current

Membrane potential (mV)

C

Normalized tail current

Membrane potential (mV)

D

Normalized tail current

Membrane potential (mV)
Figure 5-6: Time-dependence of normalized HERG tail current in a HERG transfected HEK293 cell taken over 3-min intervals for 12 min.

Cells were held at -80 mV and depolarised to voltages between -60 and +50 mV for 2 s, and then clamped to -50 mV for 4 s. **Panel A:** The normalized HERG current at 0 min and after 3 min. No significant change in the currents is detected. **Panel B:** The normalized HERG current at 0 min and after 6 min. **Panel C:** The normalized HERG current at 0 min and after 9 min. **Panel D:** The normalized HERG current at 0 min and after 12 min. All data represents mean ($n = 4$) ± SEM.
Figure 5-7: PDA-mediated effects on HERG K⁺ currents expressed in HEK293 cells.

Cells were held at -80 mV and depolarised to voltages between -60 and +50 mV for 2 s, and then clamped to -50 mV for 4 s. Panel A: Control cell prior to exposure to 3 μM PDA elicited by protocol outlined in 1D. Panel B: Currents 3 min after exposure to PDA in the same cell as panel A.
Figure 5-8: Effect of PDA (3 μM) on the activation curve of HERG tail currents in HERG transfected HEK293 cells.

Currents were recorded 3 min after PDA application using the protocol in Figure 5-3. Data represents mean ± SEM (n = 4). * Denotes significantly different from control (P < 0.05, ANOVA, followed by Bonferroni post test).
5.4.2 The effect of PKC [19-36] on HERG K+ channel tail currents

The PKC inhibitor peptide PKC [19-36] was tested at 1 µM in the presence of PDA (1 µM). There was no change in the reduction of the HERG K+ current caused by PDA. To determine whether an increase in the concentration of the inhibitor peptide reduces PDA’s effect on HERG currents, 20 µM was tested in the presence of PDA. It did not affect the PDA effect in reducing HERG K+ currents. The changes in HERG currents due to PDA exposure in the presence and absence of PKC [19-36] were also examined (Figure 5-14, Figure 5-15). There was no significant change in the activation curve due to PDA in the presence and absence of PKC [19-36] in data normalized to maximum current in control cells prior to PDA exposure. To determine whether PKC [19-36] has an effect on HERG tail currents, the peptide (20 µM) was tested using the voltage clamp protocol (Figure 5-16). The peptide did not cause any changes to the tail current over time.
Fig 5-9

A - 60 mV

B - 10 mV

C + 50 mV
Figure 5-9: Effect of PDA (1 µM) on HERG K+ channel currents in the presence of the PKC inhibitor peptide, PKC [19-36] (1 µM) measured in a single HERG transfected HEK293 cells using the protocol outlined in Figure 2-12.

Each individual panel represent current measured at the applied voltage at the time-interval indicated above it. **Panel A**: The effect of PDA on HERG tail currents in the presence of PKC [19-36] at -60 mV over 3 min intervals for 12 min. **Panel B**: The effect of PDA on HERG tail currents in the presence of PKC [19-36] at -10 mV over 3 min intervals for 12 min. **Panel C**: The effect of PDA on HERG tail currents in the presence of PKC [19-36] at +50 mV over 3 min intervals for 12 min.
Fig 5-10

Effect of PDA (1 μM) on HERG currents in presence of PKC 19-36 peptide (1 μM)

A

Control

+50

-80

-60

-50

B

2.0

nA

1.0

0.5

0.0

nA

3min

C

6min

2.0

nA

1.0

0.5

0.0

nA

D

9min

nA

E

12min

nA
Figure 5-10: Effect of the PKC inhibitor peptide, PKC [19-36] (1µM) on HERG K+ currents in a single HERG transfected HEK293 cell.

The peptide was tested in the presence of PDA (1 µM) over 12 min in 3 min intervals. **Panel A**: HERG currents obtained using the protocol inset before the addition of PDA (1 µM). **Panel B**: HERG currents obtained using the protocol inset 3 min after the addition of PDA (1 µM). **Panel C**: HERG currents obtained using the protocol inset 6 min after the addition of PDA (1 µM). **Panel D**: HERG currents obtained using the protocol inset 9 min after the addition of PDA (1 µM). **Panel E**: HERG currents obtained using the protocol inset 12 min after the addition of PDA (1 µM).
Fig 5-11

Effect of PDA (1μM) in the presence of PKC 19-36 peptide (1μM)

A

Normalized Tail Current

Control

3min

Membrane potential (mV)

B

Normalized Tail Current

Control

6min

Membrane potential (mV)

C

Normalized Tail Current

Control

9min

Membrane potential (mV)

D

Normalized Tail Current

Control

12min

Membrane potential (mV)
Figure 5-11: Effect of the PKC inhibitor peptide, PKC [19-36] (1 μM) on HERG K+ currents in HERG transfected HEK293 cells.

The peptide was tested in the presence of PDA (1 μM) over 12 min in 3 min intervals. **Panel A:** Activation curve of HERG currents before and 3 min after the addition of PDA (1 μM). **Panel B:** Activation curve of HERG currents before and 6 min after the addition of PDA (1 μM). **Panel C:** Activation curve of HERG currents before and 9 min after the addition of PDA (1 μM). **Panel D:** Activation curve of HERG currents before and 12 min after the addition of PDA (1 μM). All data represent the mean (n = 4) ± SEM. * Denotes significantly different to control (P < 0.05, ANOVA, followed by Bonferroni post test).
Effect of PDA (1 µM) on HERG currents in the presence of PKC 19-36 peptide (20 µM)

Fig 5-12
Figure 5-12: Effect of the PKC inhibitor peptide, PKC [19-36] (20 µM) on HERG K⁺ currents in a single HERG transfected HEK293 cell.

The peptide was tested in the presence of PDA (1 µM) over 12 min in 3 min intervals. **Panel A**: HERG currents obtained using the protocol inset before the addition of PDA (1 µM). **Panel B**: HERG currents obtained using the protocol inset 3 min after the addition of PDA (1 µM). **Panel C**: HERG currents obtained using the protocol inset 6 min after the addition of PDA (1 µM). **Panel D**: HERG currents obtained using the protocol inset 12 min after the addition of PDA (1 µM). **Panel E**: HERG currents obtained using the protocol inset 12 min after the addition of PDA (1 µM).
Fig 5-13

Effect of PDA (1μM) in the presence of PKC 19-36 peptide (20μM)

A

Normalized Tail Current vs. Membrane potential (mV)

- Control
- 3min

B

Normalized Tail Current vs. Membrane potential (mV)

- Control
- 6min

C

Normalized Tail Current vs. Membrane potential (mV)

- Control
- 9min

D

Normalized Tail Current vs. Membrane potential (mV)

- Control
- 12min
Figure 5-13: Effect of the PKC inhibitor peptide, PKC [19-36] (20 μM) on HERG K⁺ currents in HERG transfected HEK293 cells.

The peptide was tested in the presence of PDA (1 μM) over 12 min in 3 min intervals. **Panel A:** Activation curve of HERG currents before and 3 min after the addition of PDA (1 μM). **Panel B:** Activation curve of HERG currents before and 6 min after the addition of PDA (1 μM). **Panel C:** Activation curve of HERG currents before and 9 min after the addition of PDA (1 μM). **Panel D:** Activation curve of HERG currents before and 12 min after the addition of PDA (1 μM). All data represent the mean (n = 4) ± SEM. * Denotes significantly different to control (P < 0.05, ANOVA, followed by Bonferroni post test).
Comparison of the effect of PDA (1μM) on the activation curve of HERG currents in the presence and absence of PKC [19-36] (1μM)

A

3 min 1 μM PDA
- PKC [19-36]
• + PKC [19-36]

B

6 min 1 μM PDA
- PKC [19-36]
• + PKC [19-36]

C

9 min 1 μM PDA
- PKC [19-36]
• + PKC [19-36]

D

12 min 1 μM PDA
- PKC [19-36]
• + PKC [19-36]
Figure 5-14: Effect of PDA (1 µM) on HERG K+ currents in HERG transfected HEK293 cells in the presence and absence of the PKC inhibitor peptide, PKC [19-36] (1 µM).

The peptide was tested in the presence of PDA (1 µM) over 12 min in 3 min intervals.  

**Panel A:** Activation curve of HERG currents at 3 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (1 µM).  

**Panel B:** Activation curve of HERG currents at 6 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (1 µM).  

**Panel C:** Activation curve of HERG currents at 9 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (1 µM).  

**Panel D:** Activation curve of HERG currents at 12 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (1 µM). All data represent the mean (n = 5) ± SEM. Data was normalized to maximum current in control cells prior to PDA exposure.
Comparison of the effect of PDA (1μM) on the activation curve of HERG currents in the presence and absence of PKC [19-36] (20μM)

A. 3 min 1 μM PDA
   - PKC [19-36]
   - + PKC [19-36]

B. 6 min 1 μM PDA
   - PKC [19-36]
   - + PKC [19-36]

C. 9 min 1 μM PDA
   - PKC [19-36]
   - + PKC [19-36]

D. 12 min 1 μM PDA
   - PKC [19-36]
   - + PKC [19-36]
Figure 5-15: Effect of PDA (1 µM) on HERG K⁺ currents in HERG transfected HEK293 cells in the presence and absence of the PKC inhibitor peptide, PKC [19-36] (20 µM).

The peptide was tested in the presence of PDA (1 µM) over 12 min in 3 min intervals. **Panel A**: Activation curve of HERG currents at 3 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (20 µM). **Panel B**: Activation curve of HERG currents at 6 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (20 µM). **Panel C**: Activation curve of HERG currents at 9 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (20 µM). **Panel D**: Activation curve of HERG currents at 12 min after the addition of PDA (1 µM) in the presence and absence of the inhibitor peptide (20 µM). All data represent the mean (n = 3) ± SEM. Data was normalized to maximum current in control cells prior to PDA exposure.
Figure 5-16: Effect of the PKC inhibitor peptide, PKC [19-36] (20 µM) alone on HERG K⁺ currents in HERG transfected HEK293 cells. The inhibitor peptide did not have an effect on the HERG tail currents over 12 min with recordings taken at 3min intervals. Data represent mean of 4 ± SEM.
5.5 Discussion:

HERG channels contain phosphorylation sites for PKC (Thomas, 2003) and in the present study PDA which activates conventional and novel PKC isoforms altered the characteristics of HERG activation by decreasing tail currents in HERG transfected HEK293 cells. In HERG transfected Xenopus oocytes similar results were observed with another phorbol ester PMA (Thomas, 2004b; Thomas, 2003).

The question arises whether this effect actually involves PKC phosphorylation of the HERG channel since Thomas (2003) showed that when the PKC phosphorylation sites were mutated to be inactive, the effect of PMA to decrease currents was still apparent. The specific activator of conventional PKC isoforms thymeleatoxin exerts the same mechanism of action as PMA, causing a decrease in the tail currents at voltages positive to 0 mV (Thomas, 2003). These effects were still apparent in the absence of phosphorylation sites on HERG. In the present study, PDA was used to activate PKC to determine whether it regulates HERG currents in HERG transfected HEK293 cells. PDA was chosen as it is less lipophilic than PMA which is likely to be impeded from entering the cell interior by sequestration within the plasma membrane (Kotsonis, 1996; Murphy, 1999). PDA is less potent than PMA and therefore a concentration of 1 µM was used in the present experiment as in other cultured cell lines (Haynes, 2002). The results in the current study are consistent with those obtained using PMA in Xenopus oocytes where a decrease in HERG outward currents was observed (Kiehen, 1998; Thomas, 2003). However, the effect of PDA was not attenuated in the presence of the pseudo substrate PKC inhibitor, PKC [19-36].
The most obvious studies to perform are to block phorbol ester effects with PKC inhibitors. Indeed Thomas (2003) blocked PMA effects with Ro-32-0432. However, it should be noted that this drug caused a weak inhibition of the channel in the same study reported and that related compounds such as bisindolylmaleimide I have been shown to directly inhibit HERG (Thomas, 2004a), presumably because they are heterocyclic compounds fitting the pharmacophore for direct channel inhibition (Cavalli, 2002). The structural similarities between Ro-32-0432 and bisindolylmaleimide I suggest that there may be difficulties in using such inhibitors. Indeed Ro-32-0432 by itself appeared to block the channel in the study by Thomas (2003).

The present study used the peptide PKC [19-36] because it decreases the chance of direct channel inhibition and also identifies specific events. It should be noted that phorbol esters activate a range of non-PKC proteins such as ras-guanyl releasing protein (Ahmed, 1990; Areces, 1994) and chimaerins (Ebinu, 1998) which would not be affected by PKC pseudo substrate drugs. PKC [19-36] is highly potent and specific for conventional PKC (Smith, 1990) and is a strong inhibitor of the PKC pseudo substrate (Hvalby, 1994). The inhibitor peptide corresponds to a region of the regulatory domain of PKC isoforms in the calcium-dependent PKC family (House, 1987). PKC [19-36] had no inhibitory effect on PDA’s reduction of HERG currents in the current study when applied intracellularly via the patch pipette. This could be due to several reasons including non-specific actions of phorbol esters, which may activate other intermediate proteins that regulate HERG function or due to regulation of current by the novel PKC isoforms that are also activated by phorbol
esters and exempt from PKC [19-36] (Roivainen, 1993a; Roivainen, 1993b). The inhibitor peptide was tested at concentrations substantially higher than its Ki of 147 nM to ensure complete inhibition of the pseudo site. The inhibitor peptide had no effect on HERG currents obtained in the absence of PDA. This result strongly supports the possible direct effect of PDA on the HERG K+ channel or activation of novel PKC isoforms and excludes the involvement of conventional PKC.

The conclusion of the present study that PKC may not be involved in the inhibitory effects of PDA on HERG currents is at odds with the conclusions of Thomas (Thomas, 2003). There are several possible explanations. The most obvious is the type of PKC inhibitor used in each study, PKC [19 - 36] is specifically formulated to target the pseudo substrate site of PKC and is highly selective for conventional PKC isoforms (Smith, 1990). The inhibitor used by Thomas Ro 32-0432 acts on the ATP binding site of PKC and whilst it shows selectivity over other kinases as do other bisindolylmaleimides, the selectivity is only relative, see reviews by (Salamanca, 2005; Wilkinson, 1993). Furthermore it may target a wider range of PKC isoforms than PKC [19 – 36], including the novel isoforms although this has not been firmly established (Wilkinson, 1993). Another difference is that the PKC inhibitor in the present study was restricted to the cytoplasm as it was in the patch pipette and thus may have a more restricted access to PKC than small molecules such as Ro 32-0432 which can penetrate the cell membrane. This may be important as PKC translocation to the membrane is often a fundamental step in its activation. However PKC [19 – 36] has been shown to block PKC modulation of ion channel events in the whole cell
patch technique of the present study, in the concentration range used in other systems (Koumi, 1995; Xu, 2003).

Another study has shown that the PKC activator, phorbol dibutyrate enhances the rapidly delayed rectifier K⁺ current in guinea pig ventricular myocytes (Heath, 2000). This effect is totally opposite to other reported studies, as an increase in tail currents indicates a shift in the activation curve towards negative voltages. This effect might be due to another K⁺ channel encoding the delayed rectifier K⁺ current in guinea pig.

Interestingly, PDA’s effect of the HERG tail currents shows similar characteristics to known HERG K⁺ channel inhibitors such as terfenadine, halofantrine, chloroquine and mefloquine (Crumb, 2000; Roy, 1996; Traebert, 2004; Woosley, 1993) in that the phorbol ester decreased the tail currents at voltages positive to -20 mV.

In conclusion, the use of phorbol esters such as phorbol 12, 13 diacetate are not suitable for studying the regulation of HERG currents by PKC as these result in channel inhibition.
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