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Screening lead compounds for QT interval prolongation

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The late detection of cardiotoxic side effects, such as QT prolongation, induced by compounds of pharmacological interest can dramatically impede drug discovery and development projects, and consequently increase their cost. The launch of new drugs with undetected cardiotoxic side effects could have hazardous consequences and could trigger lethal cardiac dysrhythmias in patients. It is desirable, therefore, to test for the potential cardiotoxic side effects of compounds at an early stage of drug development. Electrophysiological test systems and cellular-based fluorometric high-throughput assays are now available for cloned human cardiac ion channels. These test systems are important tools in the preclinical safety evaluation of drugs and newly developed compounds.

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▼ During the past seven years, the development of several compounds has been aborted in late phases of drug development programmes because of undesirable effects on cardiac repolarization[1] [assessed in terms of the QT interval in the electrocardiogram (ECG)]. Furthermore, compounds have been withdrawn from the market because of suspected or proven cardiotoxic side effects[1]. The application of some of these compounds caused, in rare cases, a prolongation of the QT interval of the ECG (Fig. 1). The ECGs of affected patients resembled those of persons suffering from an inherited long QT syndrome (LQTS). Thus, these compounds could have the inherent property to cause polymorphic ventricular dysrhythmias, which can result in ventricular fibrillation and lead to sudden death[2].

Compounds shown to elicit a prolongation of the QT interval are found among distinct classes of compounds used in various therapeutic areas and include antiallergics, antibiotics, antidepressants, neuroleptics, antimalarials, antimycotics, antihypertensives and anticancer drugs[1]. The compounds have different chemical structures and vary in their mechanisms of action (e.g. calcium channel-, serotonin- and *N*-methyl-D-aspartate (NMDA)-receptor antagonists). In several cases, cardiotoxicity was not detected until clinical trials or pharmacological safety tests in non-rodents, such as ECG investigations in dogs and pigs, were performed. Because these tests are low throughput and high cost, they are usually performed in the late phases of drug development. Consequently, the decision to withdraw a compound from a drug development program is made at a late stage and can have a profound impact on the financial outcome of the project. It is, therefore, desirable to test for potential cardiotoxic side effects of compounds as early as possible during drug development. The Committee for Proprietary Medicinal Products (CPMP) has recommended a range of *in vivo* and *in vitro* preclinical strategies for consideration[3]. The recommendations provide a basis for the development of early HTS methods to determine the potential of drug candidates to prolong cardiac repolarization.

Over the past five years, ion channels have been cloned that are involved in cardiac repolarization[4,5]. Thus, targets that might be implicated in drug-mediated prolongation of the QT interval are available for the development of low-cost HTS systems. It is probable that screening of these potential targets for interaction with newly developed drug leads will be implemented in the next CPMP guidelines as a primary *in vitro* test system. However, cloned ion channels, expressed either transiently or stably in cultured cells, might differ in subtype composition, assembly or intracellular regulation from *in vivo* expressed channels, which has to be taken into account when HTS results are interpreted. In this review, the ion channels potentially involved in

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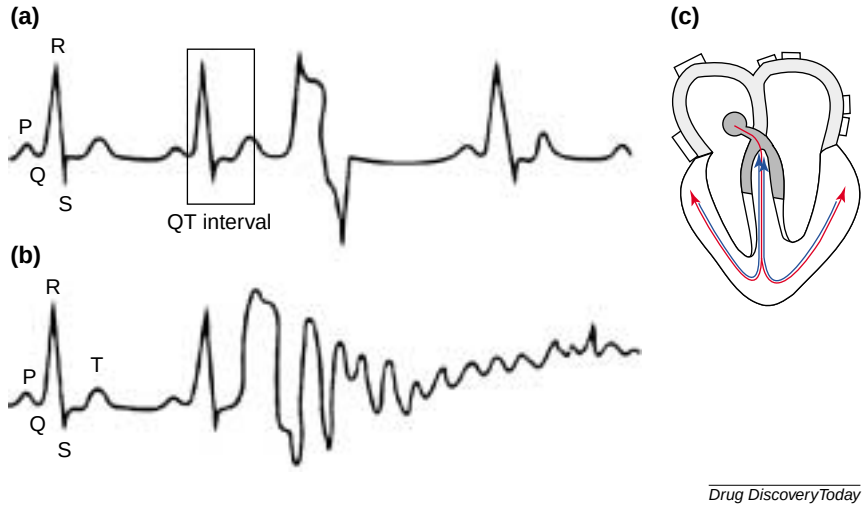


Figure 1. Electrocardiograms (ECGs) recorded from individuals with arrhythmia [ventricular extrasystole, **(a)**] and polymorphic ventricular tachycardia [torsade de pointes, **(b)**]. Standard nomenclature of the ECG is shown at the beginning of the ECG recordings. QT interval is indicated by the blue box. **(c)** Progression of normal myocardial depolarization (red) and repolarization (blue) during QT interval.

QT prolongation are described. In addition, an overview is provided that compares existing and novel cardiotoxicity screening systems.

LQTS and QT prolongation

The ECG reflects the electrical activities of atrial depolarization, and ventricular depolarization and repolarization during a heartbeat. The signal can be divided into P, Q, R, S and T phases (Fig. 1). The QT interval corresponds to the depolarization and repolarization of the ventricular myocytes. A prolongation of the QT interval is clinically characterized by polymorphic ventricular tachyarrhythmias (torsade de pointes) and can evoke syncope or sudden death. Prolonged QT intervals represent an increase in ventricular action potential (AP) duration at the cellular level[6]. The shape of a cardiac AP reflects the summation of voltage changes across the cellular membrane, which are generated by the influx and efflux of ions. Ion pumps, ion exchangers and, most importantly, voltage-gated ion channels permit the flux of ions across the membrane[7]. The activities of voltage-gated sodium and calcium channels mainly determine the depolarizing inward currents, and the different voltage-gated potassium channels (K_v channels) determine the outward currents that repolarize the cells of the human heart[8].

The following K_v channels are expressed in human heart:

- KCNA5 (K_v1.5);
- KCNB2–KCNF2 (K_v2.1–K_v6.2);
- KCND3 (K_v4.3);
- KCNQ1–KCNE1 (K_vLQT1–MinK); and
- KCNH2–KCNE2 (HERG–MiRP).

Of these, the heteromultimeric K_vLQT1–MinK and HERG–MiRP potassium channels appear to play the most important role in QT interval regulation[7] (Table 1) for the following reasons:

(1) I_{Kr} currents mediated by HERG–MiRP channels and I_{Ks} currents mediated by K_vLQT1–MinK channels are prominent during late phases of the cardiac AP and contribute to its termination.

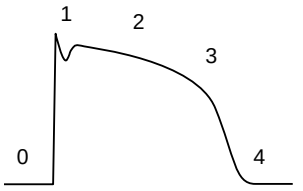
(2) The KCNE1, KCNE2, KCNH2, and KCNQ1 genes correspond to four out of the six chromosomal loci, (LQT1 to LQT6), associated with hereditary LQTS[9] (Table 2).

(3) In electrophysiological investigations, many compounds known to prolong QT interval have been shown to block HERG and/or HERG–MiRP channels expressed in cultured cells.

KCNH2–KCNE2 (HERG–MiRP) potassium channels

The human ether-à-go-go-related gene (HERG)[10] encodes the potassium channel that provides the major repolarizing current early in phase 3 of the cardiac AP (Table 1). HERG channels can associate with auxiliary MiRP subunits[11], which might affect the gating and pharmacological properties of HERG channels. However, it is not clear whether all cardiac HERG channels represent a hetero-oligomeric assembly of HERG and MiRP subunits. HERG–MiRP channels generate the I_{Kr} current that is recorded from isolated cardiomyocytes. The gating behaviour of cloned HERG and HERG–MiRP channels expressed in *in vitro* expression systems is unusual for K_v channels[11]; upon depolarization to positive membrane potentials, the HERG and HERG–MiRP channels inactivate more quickly than they activate. During the ramp repolarization at the end of an AP, the inactivated channels recover and an outward current is observed[12]. This current supports the early repolarization of the heart cells. HERG and HERG–MiRP channel activities are modulated by extracellular potassium[13]; lowering the extracellular potassium concentration reduces HERG channel-mediated outward flux of potassium ions. The potassium dependence of HERG channel activity has been correlated with the observation that hypokalaemic patients sometimes exhibit a prolonged QT interval. Furthermore, several compounds developed for

Table 1. Participation of potassium channels in human ventricular cardiac action potential



Current	Channel subtype	Phase
I_{Ksus}	K_v2.1–6.2	1,2
I_{Kur}	K_v1.5	1,2
I_{to}	K_v4.3	1
I_{Ks}	K_VLQT1–MinK	2,3
I_{Kr}	HERG–MiRP	3

Abbreviations: I_K, voltage-gated potassium current; K_v, voltage-gated potassium channel; sus, sustained K current; ur, ultrarapid; to, transient outward; s, slow; r, rapid.

various therapeutic areas that have been shown to lead, in rare cases, to a prolongation of the QT interval[14] (e.g. the antihistaminic compounds terfenadine, astemizole and loratadine), are also known to block HERG and/or HERG–MiRP channels expressed *in vitro* in mammalian cells and *Xenopus* oocytes[15]. Correspondingly, more than 50 mutations in the KCNH2 and KCNE2 genes have been described that are associated with inherited LQT disorders (http:www.ssi.dk/en/forskning/lqtsdb/herg.htm; http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?152427).

KCNQ1–KCNE1 (K_VLQT1–MinK) potassium channels

The K_VLQT1–MinK channels mediate currents that are activated very slowly with time. Maximal currents are induced by depolarizing stimuli in seconds[16,17]. The distinct properties of K_VLQT1–MinK channels account for the I_{Ks} currents, which represent a major repolarizing current during phase 3 at the end of the cardiac AP (Table 1). Mutations in the KCNQ1 and KCNE1 genes have been studied extensively; more than 80 mutations have been described that are linked to loci LQT1 (KCNQ1) and LQT5 (KCNE1; Ref. 18). Potentially, blocking of K_VLQT1–MinK channels can also cause prolongation of the QT interval. However, recent results from pharmacology screening tests suggest that compounds block HERG and/or HERG–MiRP channels more frequently, and with higher potency, compared with K_VLQT1–MinK channels.

Table 2. Chromosomal loci of inherited long QT syndrome (LQTS)

Locus	Gene	Channel	Ion
LQT1	KCNQ1	K_VLQT1[a]	K^+
LQT2	KCNH2	HERG[b]	K^+
LQT3	SCN5A	Cardiac Na^+ channel	Na^+
LQT4	?	?	?
LQT5	KCNE1	MinK[a]	K^+
LQT6	KCNE2	MiRP[b]	K^+

[a]K_VLQT1 and MinK can associate to form heteromultimers.
[b]HERG and MiRP can associate to form heteromultimers.

Screening methods to investigate the potential of compounds to prolong the QT interval

Several screening systems are available to investigate the potential of compounds to prolong the QT interval. The CPMP approved some in 1997 (Ref. 3) and others have been developed more recently. The available screening systems, however, differ markedly with respect to time, cost and information obtained. Here, common screening methods are briefly described and compared.

In vivo *investigations of cardiotoxicity of compounds in non-rodents*

In vivo experiments investigating the cardiotoxicity of compounds are typically performed on conscious or anaesthetized dogs[19]. In addition, several animal models for arrhythmogenic effects of compounds, such as those in guinea pigs and rabbits, have been described[20,21]. During application of the compounds, parameters such as heart rate, blood pressure and ECG (particularly QT interval) are measured[19]. A major advantage of these experiments is that data are generated under physiological conditions. This method has proved essential for the preclinical

characterization of compounds and has been used for several years by pharmaceutical companies and universities. Consequently, many compounds have been characterized using this experimental system and can now serve as controls in *in vitro* systems[22]. However, it has to be taken into account that the expression of cardiac ion channels can differ between humans and other species. Large variations in the shape of the cardiac AP and in the beating frequency[23] are therefore observed. In addition to this species difference, high cost and low throughput limit the application of this methodology. Moreover, access to suitable animals for such investigation is severely limited in several countries. Therefore, we conclude that this type of screening system should be reserved for compounds that are advanced in the developmental process.

In vitro *electrophysiology using primary cardiac tissue*

In vitro electrophysiological experiments in primary cardiac tissue are performed on Purkinje fibres and on papillary muscle cells of various species, such as guinea pig, rabbit, dog, sheep and pig. During the electrophysiological measurements, the duration of APs (generally measured as $APD_{50/90}$), their amplitude (APA), depolarization rate (V_{max}) and the resting membrane potentials of the cells are recorded[6]. Compared with the *in vivo* experiments, this system has the advantage of measuring the effect of a compound on all ion transport systems that contribute to the AP shape in the tissue of an animal. However, the high frequency of spontaneous APs detected in several species, such as the rabbit, necessitates pretreatment of the tissue with elevated concentrations of potassium, which might modify some of the physiological ion exchangers or channels. In addition, if compounds interact with two or more of the ion channels involved in forming the AP (Na^+, K^+ or Ca^{2+} channels), a bell-shaped concentration–response relationship is observed. Therefore, a prolongation can only be detected at specific concentrations and the experiments have to be performed at a broad range of concentrations, which is both time intensive and low throughput. To establish which of the contributing ion channels induces the bell-shaped curve, further experiments with isolated currents have to be performed. Comparable with the animal experiments, large variations between species can be observed[23]. Therefore, guidelines that recommend suitable species for these experiments would be of value.

In vitro *electrophysiology using* Xenopus *oocytes or cultured cells expressing cloned ion channels*

For experiments in *Xenopus* oocytes, *in vitro* transcribed mRNA encoding human ion channels is injected into the cells. In experiments using cultured cells, vectors containing the gene for the ion channel of interest are transiently or stably transfected into cells. Two-electrode voltage-clamp experiments are performed with the oocytes and patch-clamp experiments with the cultured cells. The currents mediated by the expressed ion-channel subunits and the effects of test compounds on these currents are investigated[24]. The advantages of this method compared with the methods previously described are the significantly lower cost and the precise characterization of the effects of compounds on a defined ion channel. However, there are pronounced differences between investigations in the two expression systems. Investigation of the effect of a compound using oocytes is less sensitive compared with native or transfected cells. The concentration–response relationship obtained with this method is typically shifted by as much as 100-fold to higher values compared with primary or transfected cells[10,12,25] and can vary depending on the preparation of the oocytes (e.g. peeled or unpeeled). Furthermore, oocytes cannot be studied easily at physiological temperatures (i.e. 37°C). The disadvantages associated with oocyte experiments do not occur during patch-clamp examinations in cultured cells. For tissue culture experiments, an appropriate cell line has to be selected. For example, human embryonic kidney cells (HEK 293), in contrast to Chinese hamster ovary (CHO) cells, express endogenous potassium channels[26] and are therefore less suitable for the investigation of potassium currents, such as HERG-mediated currents. The time required for electrophysiological investigations in oocytes and cultured cells makes HTS of compounds unfeasible at present. In recent years, automated patch-clamp systems have been developed and installed in several laboratories. It is not known yet whether the quality and reliability of the investigations using automated systems will be sufficient for effective evaluation of the electrophysiological effects of a drug. Meanwhile, it will require more progress to increase the throughput of screening to a level that is similar to, for example, fluorescence-based assays.

Binding competition assays

Cardiac muscle cells, stably transfected cell lines or membrane preparations thereof can be used for competition binding experiments with ^{3}H-labeled dofetilide[27]. Dofetilide is a potent blocker of the HERG-mediated potassium current. After saturation of HERG channels with ^{3}H-labeled dofetilide, the compound of interest is incubated with the dofetilide–channel complex and then competes with dofetilide for binding to HERG. The advantage of this method compared with the test systems discussed previously is its low cost and high throughput. By contrast, compounds that do not compete with dofetilide, but block

HERG-mediated currents will not be detected using this method. It is probable that there is a multitude of sites for blocking HERG function because there is many compounds of different chemical classes that block HERG. Furthermore, the competition-binding test screens for the binding activity of the compounds, but does not demonstrate any resultant alteration in electrophysiological recordings, thus suggesting only a putative effect on HERG functionality.

Fluorometric assays with cells stably transfected with ion channels

Fluorescence assays are used in various HTS methodologies. To test for a possible interaction with, for example, HERG, cultured cells stably transfected with this ion channel are grown in multi-well plates and are used to perform fluorometric assays. These assays exploit the properties of potentiometric fluorescent dyes that, upon alteration of the cell membrane potential, either:

- redistribute from the outside to the inside of the cells (or vice versa), which is correlated with an alteration in fluorescence intensity[28] or
- flip between the inner and outer side of the membrane bilayer, thereby enabling the dyes to perform fluorescence resonance energy transfer (FRET) with dyes at the outer side of the membrane[29].

These assays detect functional agonistic or antagonistic interaction of compounds with HERG or K_vLQT1–MinK potassium channels because a change in K_v channel activity is associated with a change in membrane potential. The automated pipetting and the short read-out time of the assays (e.g. GENION's HERG-assay: 384 wells in ~30 sec) is sufficiently rapid for HTS of compounds. Data obtained with cell-based fluorescence assays using membrane potential-sensitive fluorescent dyes are in agreement with those from patch-clamp experiments (Fig. 2). However, the results of use- or voltage-dependent antagonists might display larger differences compared with electrophysiological methods. Although no detailed information on channel–compound interaction comparable with electrophysiological recordings can be obtained, the lower cost and higher throughput of these assays makes them an alternative to electrophysiological and other test systems for screening a large number

Table 3. *In vivo* and *in vitro* methods for the screening of drug cardiotoxicity

Method	Species	Parameters measured	Advantages	Disadvantages
In vitro electrophysiology	Dog, pig, rabbit, guinea pig	Heart rate, blood pressure, ECG	Similar to physiological situation, generates a large amount of data, reproducible	High cost, low throughput
In vitro electrophysiology (Purkinje fibres and papillary muscle)	Dog, pig, rabbit sheep, guinea pig	$APD_{50/90}$, APA, V_{max}, membrane potential	Generates a large amount of data	Poor reproducibility, moderate throughput, interspecies variation
In vitro electrophysiology in *Xenopus* oocytes	Human channels in *Xenopus* oocytes	Potassium current	Data for specific compound–channel interaction, reproducible	Quantitative variation to other methods, moderate throughput
In vitro electrophysiology in cultured cells	Human channels in cultured cells	Potassium current	Data for specific compound–channel interaction, good correlation with other methods, reproducible	Moderate throughput
In vitro binding assay (e.g. ^{3}H-labeled dofetilide)	Human channels in cultured cells	Labeled ligand binding	Low cost, high throughput	Non-functional assay, non-specific binding, only one interaction site studied, radioactivity, variation to other methods
HTS using cell-based assays	Human channel in cultured cells	Membrane potential	Low cost, high-throughput functional assay	Non-specific interactions, variation to other methods

Abbreviations: ECG, electrocardiogram; $APD_{50/90}$, action potential duration; APA, action potential amplitude; V_{max}, depolarization rate.

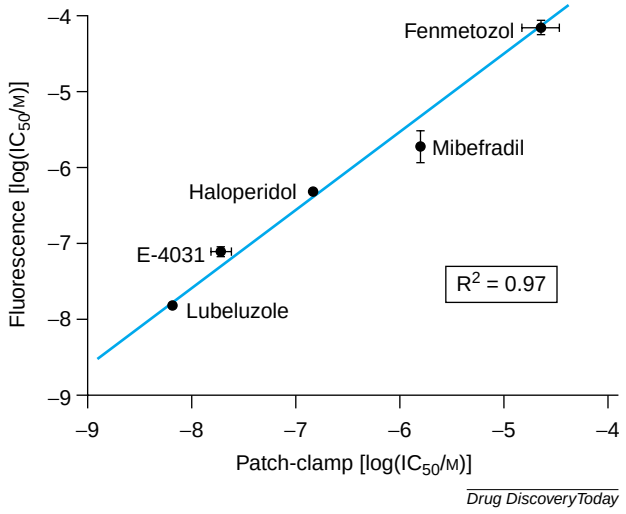


Figure 2. Correlation of the results obtained with HTS fluorescence assay and with the patch-clamp technique using Chinese hamster ovary (CHO) cells stably expressing the HERG-potassium channel. The logarithmic IC_{50} values obtained with both methods are shown. The correlation coefficient (R^2) was calculated to be 0.97, demonstrating a good correlation between the results obtained with both methods.

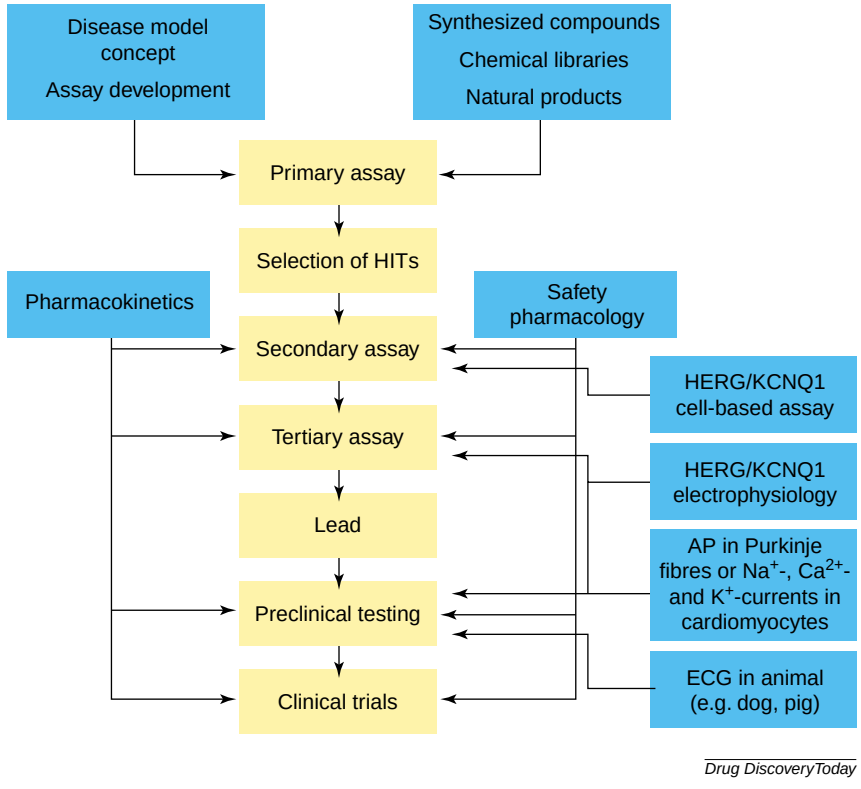


Figure 3. Proposed scheme of preclinical drug discovery and development, showing where in the discovery and development cascade different screening systems for the investigation of cardiotoxic effects could be applied.

of compounds. Table 3 summarizes the methodologies currently available for screening of compounds that potentially induce QT prolongation and cardiotoxicity.

Strategies for testing compounds for their potential to induce QT prolongation

At present, it is not possible to correlate the chemical structure of compounds with their ability to induce QT prolongation. Compounds displaying cardiotoxic side effects belong to various pharmacological groups and have disparate structures. The inability to predict the cardiotoxicity of a compound from its structure is therefore of great concern, and makes it advisable to test compounds for possible side effects as early as possible in the developmental process[8]. Currently, there is no uniform preclinical strategy used by the pharmaceutical industry to test compounds for an undesirable induction of QT prolongation. Based on the recent advances in the human genetics of LQTS, in addition to the recent development of HTS systems, we propose the following preclinical strategy to determine the potential of a drug candidate to induce QT prolongation (Fig. 3):

(1) Performance of target assay to detect hits and active concentrations.

(2) HTS using cloned HERG or HERG–MiRP channels (compound concentration ~100-fold of active concentration at target).

(3) Select 5–20 compounds to investigate the following:
- preclinical pharmacokinetics;
- electrophysiological investigation of effects on HERG- or HERG–MiRP- and K_vLQT1–MinK-mediated currents in cellular expression systems (compound concentration ~30–100-fold of estimated free plasma concentration);
- investigation of reversibility of a potential effect to detect latent accumulation; and
- concentration–response curves for HERG and K_vLQT1/MinK channels.

(4) Testing of selected compounds on Purkinje fibres, cardiomyocytes or AP profile in isolated heart preparations.

(5) Testing of selected compound on ECG *in vivo* (e.g. dog).

The summation of the results of these tests will help to recognize compounds

with a potential to induce QT prolongation early during drug development. Consequently, selected compounds will be less likely to produce cardiotoxic side effects in patients. It should be added that a stringent correlation does not exist between HERG channel blockage and cardiotoxicity of a drug; not all compounds that block HERG channels will have to be withdrawn from development or from the market. A positive risk:benefit ratio and an acceptable 'therapeutic window' are sufficient to establish a compound in the market.

Within the next few years a large number of compounds will have to be tested for potential QT prolongation using different methods. The results will be added to pharmaceutical company databases and will help to identify chemical groups and structures that should be avoided in order to develop compounds without these side effects.

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