Searching for new cardiovascular drugs: towards improved systems for drug screening?

Word count

6,203 (excluding references, figure legends, abstract and article highlight box)

Keywords

assay / cardiovascular / drugs / platform / screening / systems / technology

Abbreviations

APD	Action Potential Duration
CV	Cardiovascular
EMA	European Medicines Agency
EHT	Engineered Heart Tissue
ES	Embryonic Stem (cell)
FDA	Food and Drug Administration (USA)
HCA	High content analysis
hERG	Human ether-a-go-go related gene
hPSC	Human pluripotent stem cell
HTS	High throughput screen(ing)
iPS	Induced pluripotent stem (cell)
LQT	Long QT
MEA	Microelectrode arrays
PCT	Patent Co-operation Treaty
VF	Ventricular Fibrillation

Abstract

Introduction

The pharmaceutical industry urgently needs new ways of profiling the safety and efficacy of new cardiovascular (CV) drugs and more effectively transitioning these compounds through the stages of CV drug screening. In this article we review new technologies and methodological innovations and assess whether these frameworks offer improved solutions to the problems facing the contemporary CV drug development.

Areas covered

We performed a systematic search of the patent literature (from 2000 onwards) using US Patent Office and ESP@CENET search engines and multiple Boolean terms. We focussed on patents relating to technologies and resources and categorised the patents according to their niche in the CV drug screening landscape (molecular, cellular, organ and model organism, bioinformatics systems).

Expert Opinion

The CV drug pipeline is stalling due to the inability of many contemporary drug screening frameworks to robustly discriminate between safe, efficacious therapy and hazardous off-target effect. Given the current limitations of drug screening frameworks, there is little scope for expanding the CV drug portfolio with newer, safer drugs with improved mechanisms of action. New drug screening modalities are urgently needed. Our searches reveal that there are few examples of truly new technologies and systems in the patent literature. This apparent failure to fundamentally revamp facets of the CV drug screening process may serve only to perpetuate the inability of current platforms to improve the CV drug pipeline. Consequently, with some exceptions such as stem-cell derived cardiomyocytes, cell engineering and the emergence of phenotypic screening, there is stagnation in pre-clinical assay design that limits the pharmaceutical industry's ability to search for new drugs in new and more effective ways.

Article Highlight Box

- There is an unacceptably high attrition of CV drugs in safety assessments that stems from the failure of existing screening frameworks to appropriately assess the hazards (and as a corollary, identify beneficial off target effects) of new compounds.
- The pharmaceutical industry desperately needs new ways of screening drugs and better transitioning of lead compounds through the drug development process.
- In the article, we review recent patents in technologies and methods development in CV drug screening, covering the stages of molecules to cells, cells to syncytia, syncytia to organs and model systems, and model systems to the human scenario.
- Aside from the phenomenal progress in stem-cell technologies, tissue engineering and the emergence of phenotypic screens, we found few examples of truly innovation in the CV drug screening patent literature.
- We argue that by employing strategies that are essentially refinements of decades-old technologies, the same types of candidate molecules will be unearthed that exhibit the same hazard-to-benefit profiles as existing pharmacologies.
- We anticipate that the paucity of new modalities for improved safety and efficacy profiling will perpetuate the inability of the CV drug development process to fully assess the torrent of new candidate molecules that are emerging from high-throughput, automated lead compound discovery.

1. Introduction

In 2008, we focussed on the status of cardiovascular (CV) drug discovery and development in industrial and academic environments [1]. Although there is a general downward trend in the approval of new medical entities (NMEs) [2] that contrasts sharply with the huge pharmaceutical industry investment in R&D, this situation is critical in the CV sphere where remarkably few new cardiac drugs are emerging from the development pipeline. New drugs with innovative and plausible mechanisms of action are being brought through the system [1, 3-6] but in disappointingly small numbers that are disproportionate to the enormous disease burden of CV disease. In the wake of the high-profile problems associated with COX-2 inhibitors (rofecoxib (Vioxx®, Merck), valdecoxib (Bextra®, Searle & Company), celecoxib (Celebrex®, Pfizer)) [7, 8] and more recently the PPARγ ligands (e.g. rosiglitazone (Avandia®, GSK)) [9, 10], the pharmaceutical sector was sensitised to 'safety versus efficacy' issues. With a few exceptions, industry appeared to be re-purposing and refining pre-existing concepts rather than focussing on *ab initio* drug development since this strategy negates the exorbitant cost and chronicity of bringing drugs to market (presently estimated to cost in excess of $800million and take an average of 15 years for a single drug)[11]. The reversion to re-positioning apparently safe drugs with demonstrated efficacy in other therapeutic settings into the CV arena was contrasted by the remarkably innovative and novel solutions offered by the academic and smaller enterprises sector [1]. This paradox between innovative drug discovery and the anaemic CV drug pipeline, although clearly impacted by prohibitive cost and time issues, may point towards the pharmaceutical industry's inability to select and steer better and safer compounds, in sufficient number, through the labyrinthine world of drug screening. Consequently, the unacceptably high attrition of CV drugs in pre-clinical safety assessments is due in part to the failure of existing screening frameworks to appropriately assess the hazards (and as a corollary, identify serendipidous off-target benefits) of new compounds. Our conclusion was that the pharmaceutical industry desperately needed new ways

of screening drugs and more effectively transitioning these compounds through the stages of the drug development pipeline.

In this article we provide an objective review of developments in drug screening modalities covering genetic, molecular, cellular and animal model systems. We assess whether these advances offer improved solutions to the problems facing the contemporary CV drug development.

2. Review criteria

Previously, we reviewed patent documents that specifically described the development of new CV pharmacologies [1]. We included filings of interest that spanned the range from Patent Co-operation Treaty filings (PCT) through to granted patents and we took this wide-ranging approach in order to assess the levels of grass roots innovation in CV pharmacology. Indeed, at the time of writing only 3 were full patents. In the intervening three-plus years since 2008, from our coverage of 45 patent filings that were at application stage (PCT to 'A' status), 11 have now been granted full status (24%) and 13 (29%) are still under consideration. However, 15 filings (33%) have since been withdrawn. Although there are multiple reasons for this 'drop-out', including the high costs of remaining in the system, it affirms our belief that innovation is not being efficiently transposed to market development. The unpredictable nature of the outcome of many patent applications prompted us to confine our search in the present article to only those that had been granted full patent status (i.e. 'B').

Patents and their archival databases are notoriously opaque; keywords and search terms are frequently secreted away in obscure portions of the text and thus comprehensive searching of the patent literature is difficult. Some documents are wilfully vague presumably in order to avoid detailed searches. To tackle this issue, we performed a systematic search of the patent literature (from 2000 onwards) through the US patent office [12] and ESP@CENET (that indexes both European and US patents) [13] search engines using multiple Boolean terms

defined in Table 1. Search hits were refined according to the scheme described in Figure 1, and we excluded any document that related specifically to natural and synthetic pharmacologies, medicinal agents and hardware (medical devices) since these fell outside of the present remit to evaluate screening technologies and tools. We also excluded any documents relating to DNA sequences of mutant ion channels, since with some rare exceptions (e.g. a novel tension dependent channel [14]), patents in this area are primarily adding to the catalogue of disease-linked mutations in already reported genetic loci. We did however consider patents relating to newly-identified common DNA variants (polymorphisms) associated with arrhythmias since this is a field of burgeoning interest [15, 16].

Table 1 shows that our two complementary search modes (full-text searching (US Patents) and abstract and title-type searching (ESP@CENET)) yielded comparable coverage of the search terms. We classified the selected patents into model organisms, cell systems, sequencing and bioinformatics and screening technologies (Figure 1) and used these as the basis to structure the present article.

3. The need to redefine screening technologies

The pharmaceutical industry utilises an array of pre-clinical screening approaches to profile CV drug safety and efficacy. These include sophisticated technological platforms such as automated patch clamping, microelectrode arrays (MEAs) and advanced microscopy for enhanced cellular imaging. These approaches are complemented by lower throughput 'conventional' *in vitro* testing that include Purkinje fibre-based action potential duration (APD) assays, canine wedge preparations and Langendorff perfused rabbit hearts and evaluation using *in vivo* models of arrhythmia (animal models of chronic atrio-ventricular block, failing rabbit hearts, paced canine hearts). These and other contemporary drug screening modalities have been recently reviewed [17-19] (see also Table 2). The combined outputs from these systems are used to provide an integrated assessment of drug safety and

efficacy in line with current European and US regulatory mandates [20, 21]. Notably, the shortcomings of these outdated guidelines have been exposed. For example, they do not cover the potential of drugs to shorten the QT interval that may exacerbate arrhythmias and it has been argued that these criteria need to be overhauled [22, 23]. We consider this issue further in Section 4 (From molecules to cells).

The early stages of CV drug screening involve molecular and cellular profiling of lead compounds and here there is a pervasive drive towards automation, miniaturisation, and nanotechnologies that enable ever-higher throughputs to be achieved. Impressive as these technological achievements are, many of these new platforms are prone to the same problems that plague their lower-throughput configurations (e.g. cell quality, fragility of membranes in automated patch clamping [24]). Moreover, from an industrial perspective it has been suggested that too many of these assay types are prone to artefact and bias [19]. Importantly, there is a fundamental issue that directly impacts on the ability to search for new drugs in new ways - the basis of these assays has remained unchanged for decades. The perception of methodological stagnation, at least in terms of the paucity of patents relating to truly new platform modalities for CV drug screening is corroborated by our searches that revealed that half of the new patents describing screening technologies (Figure 1) are essentially glorified electrophysiological platforms [25-29]. One patent describes an antibody-based method for profiling cell-surface expression of cardiac ion channels or for investigating the total amount of protein within a cell [30]. Another claims that an assessment of differential gene expression profiles evoked by drug-treatment could be used in predicting pharmacotoxicity within cell populations [31]. However, both of these examples involve the adaptation of rather conventional techniques into the CV drug-screening arena and do not typify genuinely innovative solutions.

The majority of cell-based screens are designed to investigate short-term phenotypic and molecular changes. But where are those systems for interrogating more ‘slow-burning’ phenotypic changes? Subtle drug-induced changes in cardiac cell ion handling and signalling

may eventually lead to necrosis, apoptosis and autophagy over the course of many days or weeks - timescales that are incompatible with high content screening (HCS) assays. Likewise, the ability of small molecules or biologics to correct the chronic intracellular trafficking defects associated with many mutations in the human ether-a-go-go channel gene (*hERG*) (channelopathic mutants that underpin long QT syndrome (LQTS) type 2) is unlikely to be robustly examined by current HCS platforms. Thus, systems for interrogating longer-term effects of drug exposure (e.g. latent toxicity) and other more gradual phenotypic changes have yet to be properly configured.

So, the persistently high attrition of CV drugs in the development pipeline directly suggests that contemporary drug screening frameworks are not sufficiently discriminatory to drive the discovery of newer, safer drugs with improved mechanisms of action. We argue that by employing strategies that are essentially refinements of decades-old technologies, the same types of candidate molecules will be unearthed that exhibit the same hazard-to-benefit profiles and the development pipeline will stagnate. Innovative screening platforms are urgently required.

4. From molecules to cells: moving towards phenotypic screening strategies

As was noted over a decade ago, "molecularly orientated studies dealing with transcript determinations, gene actions and interactions, protein-protein interactions and signal transduction pathways, although obviously valuable in their own rights, fail to define over the lifetimes of the animals the changes that occur at the whole organ and whole animal levels" [32]. While molecule-centric approaches are valid in diseases with established culprits, in complex disorders such as CV disease that may be characterised by the collectivised abnormalities of ion channels, regulatory proteins and genetic and epigenetic factors, it is very difficult to identify drugable targets. Even in those instances in which cardiac disease may be attributable to discrete molecular dysfunction (e.g. monogenic ion channeopathies) there are problems

associated with this approach. For example, determining the liability of a drug to block hERG, the channel that mediates important repolarization current in humans (IK_r) is a mandatory requirement of FDA and EMA guidelines [20, 21]. However, specifically configuring a molecular screen to identify lead compounds that do not block hERG is of limited value in defining their likely hazards in a clinical setting because human arrhythmicity is governed by currents other than those carried by hERG [33]. Furthermore, the relationship between hERG blockade and arrhythmia is complex since many drugs that block hERG do not cause arrhythmias (e.g. verapamil, amiodarone) while some agents that do not directly block hERG are potent arrhythmogens (e.g. arsenic trioxide) [33-35]. Thus, such a molecular-centric assay may conspicuously fail to identify the hazardous or beneficial off-target effects of the drug under test. The impact of such issues in the CV drug pipeline is substantial; it is estimated that 40-70% of new chemical entities are abandoned because of hERG liability leading to calls that many of these 'failures' should be re-evaluated in improved assays [34, 36].

So, considering the limitations of some molecularly-centric assays, for example HERG liability which is considered by some to be a poor surrogate for arrhythmogenic liability *in vivo* [37], there is value in moving towards phenotype-based screening modes that identify lead compounds that, as Peal and colleagues eloquently phrased, "modify the disease trait in a mechanistically agnostic fashion" [38]. This is allied to the concept of 'magic shotguns' (not bullets!) through which improved clinical efficacy is driven by pleiotropic agents that act on multiple cellular targets [39]. The utility of model organisms for phenotypic screening of CV drugs is discussed in Section 8 (From organs to model organisms).

5. From molecules to cells - the stem-cell revolution

The heterologous expression of recombinant proteins in mammalian cells (e.g. CHO, HEK, HeLa) is a powerful tool for the dissection of molecular function. Whilst these systems provide a more native background for the study of ion channels than non-mammalian ones (e.g.

Xenopus) not least in their dose-response profile to drug dosing [24], they fail to recreate the hallmark functional, contractile and electrical properties of cardiac cells. Ultimately these non-cardiac 'industrial' cell types bear little physiological relevance to the environment in which the drugs will ultimately be expected to work. Their use is prone to false positives (safe drugs that appear hazardous) and the more common false negatives (hazardous drugs that have no overt dysfunctional properties).

A solution to some of these issues is presented by the method of generating beating heart cell clusters from neonatal hearts that can be used for investigating the effects of drugs on heart rate and rhythm (i.e. a phenotype screen) [40]. This technology enables the use of virally-transduced animal cells to reproduce sinus node cardiac pacemaker function that may be combined with voltage- and Ca^{2+}-sensitive dyes to monitor cellular beat rate.

Clearly, a screening strategy based on human cardiac cells obviates the species-specific problems that plague the use of cells derived from mouse, rat, rabbit and dog. The phenomenal conceptual and technical progress that has been accomplished in human pluripotent stem cells (hPSC, a term that encompasses both embryonic stem cells (ES) and induced pluripotent stem cells (iPS)) has seen their emergence as genuinely new cell platforms for CV drug screening. The stunning technical feat of reprogramming mouse fibroblasts to iPS cells using virally-driven expression of four cardiopoetic factors (Oct4, Sox2, Klf4 and c-Myc) [41] was soon followed by the generation of iPS cells from adult human fibroblasts using the same factors [42] or a modified protocol (Oct4, Sox2, Nanog and LIN28) [43] and also from foetal and neonatal fibroblasts [44]. An important development came from Zhang and colleagues who reported a methodology for obtaining functionally competent cardiomyocytes from human iPS cells [45], although it is acknowledged that efficient and reproducible differentiation into cardiomyocytes remains a challenge. In our searches, we did not find any patents relating specifically to ES-derived cardiomyocytes since there is a legal preclusion to the granting of a patent to any material that has involved the destruction of an

embryo [46]. Notably, patenting in the iPS cell field, although not prevented by the same legislation that governs ES cells, is mired in controversy over priority due in part to the US system of "first to invent" versus the European and Japanese schemes of "first to file" [47-49]. Despite feverish levels of activity in patent applications relating to iPS technologies, we were surprised that so few patents relating specifically to iPS cells had been granted [50-52]. To illustrate this, Shinya Yamanaka, whose group first generated iPS cells [41] has filed twenty-eight patent applications relating to methods development in iPS cellular technologies, that includes the one granted in Japan [50]. However, at the time of preparing this article, none of them had been granted full patent status in the US. To date, almost all of the patents granted in the ES- and iPS sphere relate primarily to methodological and protocol development that optimise the processes rather than patenting the cellular entity [53-56]. Thus at present many commercial organisations have full freedom to operate in this arena. Given the breathtaking pace of progress in this field, it is entirely likely that some technologies under consideration will be obsolete before the patent is prosecuted [49] and we believe that the present legal complexities will remain for some time to come.

Subsequent refinements to reprogramming methodologies have improved the biosafety and downstream applicability of iPS cells [57, 58] and these are fuelling the anticipated utility of these cell types in cardiac regeneration strategies via autologous transplantation [59-61]. However, this therapeutic goal may be significantly delayed due to complications associated with hPSC cell types. The pluripotency of ES-derived cells leads to teratoma formation upon implantation and this limits their usefulness in this context. Elsewhere, iPS cells exhibit by aberrant epigenetic phenomena (DNA methylation, histone acetylation) and an increased frequency of somatic coding mutations that persist in the post-differentiation state [62-65].

Given these present preclusions, the most immediate natural fit for these amazing cellular resources in the drug landscape is in screening programmes [66-68]. Indeed, iPS-derived

cardiomyocytes have already been used for this purpose and it appears that pharmacobehaviour in iPS-derived cardiomyocytes is similar to that observed with human ES-derived cardiac cells [45, 69]. Consequently, huge investment is being poured into the industrial generation of ES- and iPS-derived cardiomyocytes. The commercial availability of hPSC-derived cardiac cells (e.g. GE Healthcare's Cytiva (ES-derived) and CDI's iCell (iPS-derived)) obviates the practical challenges in reproducibly deriving phenotypically competent cardiomyocytes from ES and iPS cells. Moreover, augmenting these cellular resources with approaches that incorporate genetic selection using transgenes encoding fluorescent reporters or antibiotic selectable resistance enables the expansion to bioreactor scale culture compatible with HTS. However, the phenotypic reproducibility of the cells through the manufacturing process is crucial. Not all the cells derived from hPSC protocols are functional cardiomyocytes (although methods have been described to improve the overall yield of cardiomyocytes [55, 70]) and fibroblast 'contamination' is persistent and extremely variable. Also, even cardiomyocytes derived from ES- and iPS cells are phenotypically heterogeneous remaining embryonic-like in terms of size, organisation and electrical properties and proteomic profile even after functional maturation via prolonged culture (>50 days) [45, 60, 71]. Consequently, the validity of using hPSC-derived cells as *bona fide* adult ventricular and atrial cells is open to question. Similarly, the drive towards phenotypic enrichment of a 'pure' cardiomyocyte population moves further away from the multi-cellular mosaicism that characterises the myocardium *in vivo*.

The ability to generate iPS-derived cardiomyocytes from patients affected by monogenic arrhythmia disorders (including LQT1 [72], LQT2 [73, 74] and Timothy syndrome [75]) potentially leads to a new era of drug toxicological and efficacy profiling. iPS-derived cardiomyocytes from these individuals appear to recapitulate the molecular and cellular dysfunction associated with the human disease. For example, LQTS-patient derived cardiac cells exhibit electrophysiological and gene-expression dysfunction in ventricular, atrial and

nodal cell populations [72] whereas in iPS-derived cardiomyocytes from a patient with Timothy syndrome, the disease-linked abnormalities are restricted to a ventricular-like cell population [75]. These data point to the disease-specific functional segregation of cardiomyocytes within iPS-derived populations and this remarkable phenotypic preservation will be of high value in future screens. The utility of patient-derived cardiomyocytes in drug discovery has already been demonstrated in a compound screen in which roscovotine, a cyclin-dependent kinase inhibitor, was identified as an indirect modifier of L-type Ca^{2+} channels in Timothy syndrome-derived cardiomyocytes. This is the first example of disease-linked iPS-derived cardiomyocytes being used as a new drug screening modality to identify a novel class of anti-arrhythmic [75]. Despite the substantial enthusiasm for using hPSC-derived cells in drug screening, it is unlikely that they represent a 'cure-all' for the problems that affect the CV pipeline. Clearly, there is a limit to mechanistic insights into contractile and arrhythmogenic dysfunction that can be gleaned from studying hPSC-derived cardiomyocytes (or indeed any cell type) *in vitro*. The clinical manifestation of heart disease is influenced by complex genetic, epigenetic and environmental factors as well as interaction with other organ systems [76] Accordingly, although these new cell-based screens will form a cornerstone of CV drug screening strategies over the coming years, the data that emerges from them must be interpreted in the context of outputs from other screening systems.

A recent landmark development has been the direct reprogramming of differentiated fibroblasts into cardiomyocytes without reversion to a transient pluripotent state (using Gata4, Mef2c and Tbx5) [77, 78]. Cardiomyocytes derived from differentiated fibroblasts (iCM) are reported to functionally mature over several weeks [77], but unlike hPSC they cannot be expanded in culture. Thus, iCM are best suited for screening strategies that identify factors which can reprogram endogenous fibroblasts (which represent more than half of the cell population in the adult heart) into functional cardiomyocytes [77]. Indeed, this technological advance, together with reports that negate the concept that cell transplantation is the only

way to regenerate the ageing/failing myocardium [79, 80] will likely focus next generation of cardiac drug screening towards small molecules that promote the conversion of myocardial fibroblasts directly into functional cardiomyocytes.

6. From cells to syncitia

Assays using single cardiomyocytes yield valuable mechanistic data but their utility in the context of CV drug screening is compromised by their lack of functional modulation by neighbouring cells. Gap junction-mediated electrotonic cell-to-cell interactions are integral to the trans-cellular dispersion of repolarising ion currents and thus multi-cellular platforms that enable individual cellular behaviour to be evaluated in the context of functionally coupled networks of cells are a cornerstone of appropriate testing models.

As discussed above, hPSC-derived cardiomyocytes form beating (syncytial) clusters and provide a cellular context relevant to the human scenario. However, they still fall short of recreating the tissue architecture characteristic of the situation *in vivo*. It is here that engineered heart tissue (EHT) represents a great step forward. Scaffold-enabled EHT facilitates the investigation of physiologically relevant, three dimensional electrical (dys)function to be determined in response to chemical and physical stimuli [81, 82]. Specifically, the use of EHT recreates the much greater extent of cell-to-cell-coupling characteristic of the *in vivo* scenario. These meticulous experimental setups promote directional cell alignment and coupling, and a remarkable level of ultra-structural organization and phenotypic competency in response to electrical and mechanical cues. In one example, neonatal rat cardiomyocytes are enmeshed in a mouldable fibrin matrix and over time (15-30 days) the cells remodel this hydrogel into a pseudo-cardiomyocyte muscle strip that is amenable to specialised multi-well formats [83]. Present characterisations are restricted to gross morphometric observations of rate and force (via measurable deflections of silicon posts embedded in the culture) but the coupling of EHT to improved imaging and analytical

techniques, together with methods for improving cell survival will improve their utility in high content applications [84]. Practical obstacles of EHT notwithstanding (e.g proteolytic degradation of the hydrogel, present restriction to rat cells, cellular phenotypic immaturity [81]), EHT should comprise an important part of the engineered armoury that will improve CV drug screening. However, their amenability to anything other than low-throughput applications will need to be demonstrated.

7. From syncytia to organs

Ventricular repolarization, defects of which are common in human arrhythmias, is complex and depends on the density, molecular specificity and structural arrangement of ion channels and cell-surface receptors. These factors dictate the regional heterogeneity of the myocardium [85] that governs repolarisation dispersion across multiple cellular layers and can only be properly modelled in intact organs. Purkinje fibres are widely used for assessing APD but their use for this purpose is inappropriate since these fibres do not contribute to the QT interval and they are insensitive to some weaker QT prolonging agents. Moreover, their response may also be markedly different to ventricular tissue. Elsewhere, the potential limitations of data from hPSC-derived cardiomyocytes may be difficult to interpret properly because the myocardium is composed of several cell types in defined structural organisation [71]. The niche afforded to organ studies in CV drug development is demonstrated by the finding that repolarization gradients across the heart dictate the relative pro- or anti-fibrillatory actions of Na^+ channel blockade [86]. Similarly, the efficacy of a new lead compound (AZD1035) was predicated by the foci of the arrhythmia [87]. In another study, Hondeghem screened over 700 repolarisation-delaying drugs in perfused rabbit hearts and showed that the ability to interrogate organ-based data using TRIaD (triangulation, reverse use dependence, AP instability and dispersion) revealed good pro- and anti-arrhythmogenicity indices, some of which ran counter to what may have been anticipated from non-organ (cellular) screens [88].

Our searches did not reveal any new innovation in this area of CV drug screening; whether this reflects lack of development or that current modalities are entirely fit for purpose is difficult to ascertain.

8. From organs to model organisms

As we discussed in Section 6, the complex mechanisms that underpin the initiation and progression of cardiac dysfunction *in vivo* restricts the usefulness of strategies centred on *in vitro* cell- and organ based assays in CV drug screening. The use of model organisms enables a more physiologically relevant context for the profiling of lead compounds, including the ability to investigate their effects on tissues other than the heart [89]. The utility of mouse genetic models for yielding rich mechanistic information in the context of monogenic arrhythmia disorders arising from Na^+, K^+ and Ca^{2+} ion channelopathies is well known. Such genetic models of cardiac contractile and arrhythmic disease are complemented by numerous small animal models of cardiopathology especially heart failure induced by a variety of surgical and non-surgical approaches [90]. However, from a physiologic perspective the use of mouse models as an appropriate surrogate of human cell function is fundamentally flawed; heart rate is tenfold-faster than in humans, they lack an innate propensity towards arrhythmia and there are markedly different electrophysiological bases in their cellular action potential. Consequently, these species-dependent limitations have led the pharmaceutical industry to conclude that the mouse and rat heart are not appropriate resources for testing arrhythmogenic liability [20]. Other animal models whose cardiac physiology is more closely aligned with human are considered more appropriate (e.g. dog, guinea pig, ferret, rabbit), and indeed there have been new technological developments that enable the generation of transgenic rabbits and other non-murine models of QT prolongation [91, 92]. Again, the caveat is that these do not faithfully recreate a human-like scenario.

It is perhaps surprising that in view of the need to make animal models of cardiac (dys)function as relevant to humans as possible that the zebrafish embryo model is gathering momentum as a phenotypic CV drug screening platform [93]. Despite the obvious differences in CV morphology and anatomy, and very different mechanisms of drug bioavailability that make assessments of drug toxicity and latency difficult [94], early studies in zebrafish reported findings that could plausibly be extrapolated to the human scenario [95]. The model has many advantages; embryos are transparent, easy to maintain and highly adaptable to multi-well formats (3 embryos per well for a response in triplicate!), and a contractile atrio-ventricular system is formed within 3 days post-fertilization. These attributes afford a wealth of analytical techniques and the phenotypic assessment of CV (dys)function (e.g. 2:1 atrio-ventricular block) can be augmented by the inclusion of cellular imaging using fluorescent-based techniques.

In addition, the availability of cardiopathic mutants such as *reggae* (mimic of short QT syndrome and 'pseudo'-AF via gain of function zebrafish ERG (zERG) channel mutation (L499P) [96]) and *breakdance* (QT prolongation by loss-of-function I59S zERG mutation [38]) that recapitulate some of the contractile and arrhythmogenic dysfunction characteristic of the human genetic cardiopathiologies [97] augments their applicability to CV drug screens. Indeed, mutant zebrafish have been used to identify flurandrenolide (a glucocorticoid receptor ligand) and a compound termed 2MMB (unknown mode of action) as agents that rescued beating phenotype despite not rescuing the trafficking defects of I59S zERG in *breakdance* embryos [38]. The availability of libraries of chemically-mutated zebrafish (via N-ethyl-N-nitrosourea (ENU) mutagenesis,) now facilitated by large-scale resources such as those at the Sanger Institute [98], will further enhance their utility.

Although the zebrafish model is gaining traction as a platform for early stage CV drug screening that enables the rapid phenotypic screen of a large number of compounds, their use is not without problems. Firstly, the absorption of drugs through the skin of the fish is

critically dependent on their physico-chemico properties and this makes an accurate assessment of drug potency extremely difficult. Second, the embryonic development status of the zebrafish may predispose to artefacts e.g. the action of glucocorticoid agonists/antagonists.

9. Bioinformatics

From Moller and Slack's excellent review [19], it seems that the breathless pace of funnelling huge numbers of compounds into assays of ever higher throughput and content is not (so far) yielding breakthrough therapies. There is a real risk that high throughput strategies will lead to a state of "data rich, knowledge poor". High content analysis (HCA) that comprises multi-parametric analysis of readouts from cellular imaging platforms requires high-end programming and computation to be seamlessly integrated with a fundamental knowledge of the underlying biology. Downstream deconvolution and interpretation of the direct (and indirect) outputs that come from complex high content screens remains an ongoing challenge [99].

In parallel to experimental drug screening, the pharmaceutical industry is turning to sophisticated computational and bibliometric methods for identifying novel indications for new and old drugs. Systematic statistic-based screens for drug-ligand interactions ('chemoinformatics') are yielding valuable information [100 99] and a recent article proposed the use of literature-mining methods to identify trends in drug development that map to burgeoning areas of mechanistic advance [101]. These approaches expand our horizons beyond traditional and/or predictable targets of CV drugs and are rendered more credible by the demonstration that many genetic loci linked to QT prolongation would not have been considered typical CV drug targets [102-104]. However, Agarwal's concept is dependent on isolated pockets of innovation rapidly gathering momentum and there is the possibility that

innovators of true value are lost in the noise [101]. There is also a more general concern regarding an over-reliance on databases that may be incomplete and/or biased.

10. To the human scenario

Even when the insights into CV disease gleaned from advanced molecular, cellular and *in vivo* studies are considered, no pre-clinical assay portfolio will likely be configured that replaces the value of screening lead compounds in large, well controlled clinical trials. The response of an individual or population to a particular drug regimen is so complex (and often unpredictable) that it is almost impossible to pre-empt all risk at the pre-clinical level. Thus, for all our understanding of molecular interactivity, cell-to-cell networks and pharmacogenomics, in a standard clinical setting the propensity toward ventricular fibrillation (VF) may be predicated by ischemic episodes and non-functional regions of the myocardium or pro-arrhythmic responses to systemic hypoglycaemia [105]. The common inability of pre-clinical testing to accurately predict clinical value is highlighted by Pfizer's Anthony Coyle who has stated that "In vivo validation has zero impact, in most cases, on whether you will be successful going into the clinic" [106]. Below, we consider some examples that highlight the irreplaceable need for the thorough clinical evaluation of CV drugs.

The clinical efficacy of dronedarone, a non-iodinated amiodarone derivative, is critically dependent on pre-existing cardiac disease. It is proving to be useful in the treatment of atrial fibrillation [107] but it critically exacerbates cardiac dysfunction when administered in the setting of heart failure [108]. The mechanistic basis of this idiosyncratic profile remain unknown. Similarly, the successful re-appropriation of flecainide for the management of stress-induced arrhythmia in structurally normal hearts [109] could hardly have been predicted from its exceptional hazard in the context of ischemic heart disease [110]. In both these instances, the dependence of therapeutic efficacy on the pre-existent cardiac status in the

clinical scenario poses real and often intractable problems for those configuring pre-clinical testing models.

Gender influences numerous CV-relevant phenomena including heart rate, the autonomic nervous system, electrolyte balance and the susceptibility to pharmacologic perturbation of the QT interval. The susceptibility for QT prolongation in females may result from a different density of K^+ channels that is possibly due to hormonal regulation of protein transcription/turnover. Hepatic metabolism of many drugs (e.g. β-blockers [111]) is also different between men and women leading to differential bioavailability that is difficult to predict from the non-human testing.

The concept that an individual's genome could tune the predisposition to arrhythmia or predict the ultimate benefits / hazards of a specific drug regime underlies the notion of genetic risk in the population. A genetic predisposition that re-balances a drug's safety and efficacy profile [112], may be underpinned by common DNA variants termed genetic polymorphisms. Up until recently such polymorphisms, that occur in both intronic and exonic gene regions and may or may not produce discrete changes in the translated protein sequence, were considered benign sequence variations that had negligible functional consequence. However it is emerging that common genetic polymorphisms may causally modify cardiac ion handling and impact on an individual's response to drugs [113, 114]. We are beginning to see the emergence of this complex genetic heterogeneity in the CV patenting landscape. In one example, a common polymorphism at nucleotide 3308 in the SCN5A gene (encoding the $Na_v1.5$ channel) results in an amino acid change (S1103Y) that is reported to predict torsadogenic liability [115]. It has already been shown that another polymorphism in this same channel that leads to the H558R amino acid substitution modulates channel function [16]. As we discussed above, iPS cell technology enables, in principle, drugs to be screened in the context of an individual's genotype. Whether such approaches move beyond technical possibility into a feasible industrial reality is keenly anticipated [116]. Recapitulating the

concepts from Section 6, in some instances genetically-linked cardiac dysfunction is precipitated by non-cardiac triggers (e.g. neuro-humoral activation) and thus data obtained from patient-derived iPS-cardiomyocytes must be interpreted in this context.

There are small animal models that can be used to investigate some aspects of sex difference [117, 118] and background genetic variation [119] but the limitations of these models with respect to their relevance to human ion handling is acknowledged.

11. Expert Opinion and concluding remarks

There is clearly an urgent need for improved pre-clinical surveillance systems that enable the unbiased profiling of the safety and efficacy of new CV drugs. However, the CV pharmaceutical industry is still struggling to define and come to terms with how best to achieve this. Within contemporary screening strategies there are sensible frameworks for the better management of the transition between stages of drug development [35] but these are not resulting in improved pipeline delivery. In our opinion, the way forward necessitates new modalities for screening drugs that obviates species-dependent artefact and address emergent concepts such as phenotypic screening, pharmacogenomics, latent toxicity, cardiomyocyte phenotype and regional cardiac dysfunction. Without critically re-thinking the traditional tenets of drug screening frameworks, the investment in ever-higher throughput methods for lead compound identification will yield increasing numbers of compounds that are of negligible clinical value. In this context of vast compound library screens, and in the absence of frameworks with better abilities to discriminate between safe and effective therapy and hazardous off-target effects, it is entirely possible that the attrition rate of CV drugs in the development pipeline might actually increase.

There are no easy answers to this situation. The intricacies of cardiac ion handling and its modulation by other organ systems underpin the fact that cardiac contractility, rate and rhythm are extraordinarily sensitive to pharmacologic perturbation. Thus the keystone of

next generation CV drug screening is the ability to develop new systems that properly contextualise the exquisite balance between effective therapy and hazardous side effect. Presently, there is evidence that the balance is being tipped in favour of safety, a strategy that may paralyse CV drug development. Hans-George Eichler, Senior Medical Officer at the EMA recently stated: "If people want certainty, very soon we will not have any new drugs" [120]. After all, some highly effective drugs (e.g. aspirin, amiodarone, warfarin) would never have been approved in today's arguably more 'risk-averse' environment. Conversely, using sub-optimal systems for assessing a drug's toxicological profile is fraught with risk, both human and financial. Perhaps a more proportional sense of risk-benefit profiling is necessary such that those that may benefit from a specific therapeutic regime are not disadvantaged by far-reaching "no-go" decisions [121].

In this article we have attempted to highlight the state of new technological and methodological developments relevant to CV drug discovery and validation processes using the patent literature as a barometer of innovation. From the outset, we anticipated that we would uncover new and better systems that would form a new foundation from which to launch next generation CV drug discovery and development. However, apart from the substantial momentum in the stem-cell and cell engineering fields, and the slow emergence of phenotypic screening platforms, we found little evidence of truly innovative solutions for CV drug screening technologies in the patent literature. There are glimpses of novelty, but most of the technological advances described in patents appear to refine and develop existing methods. This apparent inability (or unwillingness?) to fundamentally revamp facets of the CV drug development process may serve only to perpetuate the failure of screening platforms to improve the pipeline.

It is also worth noting that the sort of data that will emerge from new screening modalities may be incompatible with the type of validation required by current safety mandates. Here, the arguably dated and sometimes outmoded requirements of these

guidelines (for example the failure to appreciate the importance of compounds that shorten QT and place too much emphasis on blunt hERG-blocking profiles) must be updated to become entirely relevant to the way in which CV drug screening must be developed in order to reduce the presently unacceptable pre-clinical attrition and clinical problems associated with a disproportionate number of lead compounds. As a corollary, the future emergence of new technologies and resources does not guarantee that they will be appropriate tools for next generation CV drug screening. Emerging cell-based platforms, high content analysis, bioinformatics approaches and physiologic testing must be rigorously validated to demonstrate an absolute relevance and usefulness of purpose in this new era.

According to Titus Livy, "In difficult and hopeless situations the boldest plans are the safest". The pharmaceutical industry needs to be bold and resourceful if it is to reinvigorate its CV pipeline; the situation is difficult but it is not yet hopeless.

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Figure Legends

Figure. Patent search strategy and information profiling.

[a] ESP@CENET does not enable full text searching.

[b] Only those patents that had been granted (i.e. B status) were considered and any relating to natural and synthetic pharmacology, devices, medicinal agents and DNA variants (except common sequence polymorphisms) were excluded.

[c] Discrete patents that do not develop or build upon previous patent filings.

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