Preclinical assessment of cardiac toxicity

Ralf Kettenhofen and Heribert Bohlen

Axiogenesis AG, Nattermann Allee 1, 50829 Cologne, Germany

The exact prediction of the clinical behavior of drugs represents one of the most difficult duties in preclinical drug development. The use of cell-based assay systems underpins the development of many drug candidates, but owing to the artificial character of many of these systems, cell response and physiological behavior seem to be mutually exclusive.
Embryonic stem cell-derived cells represent a system that may address the disconnect between the behavior of cultured cells and cells *in situ*. While undifferentiated ES cells allow standardization, expansion and genetic manipulation, the differentiated cells provide a reflection of the normal physiological image of their primary counterpart.
We compare common models to detect cardiac toxicity with an assay system comprising *in vitro* differentiated pure cardiomyocytes.

The design of modern drug candidates is more and more focused towards the manipulation of signaling pathways in a misbehaving cell. The functional testing of desired drug action and prediction of unwanted side effects, however, developed at different rates. Whereas signaling effects can be studied in isolated assay systems, prediction of relevant side effects needs cell lines suitable for HTS. Moreover, drug development faces the obstacle of interpreting complex cellular and animal data in order to predict the potential of a given drug candidate to produce adverse effects in humans.

So far, the huge gap of cell systems from standardization as a 'must-have', physiological and organ specific behavior as a 'nice to have' and the predictability of clinical behavior has not, so far, been closed. In addition, while functional development focusing on cellular behavior is analyzed with the most modern techniques, toxicological analyses are generally confined to relatively old test strategies.

In this review we will summarize and discuss the available models that are used at different levels of preclinical studies. We will focus mainly on the analysis of *in vitro* systems to determine cardiac cytotoxicity and will present evidence that a complete toxicological model can be designed, not only by analyzing the cytotoxic and apoptotic events, but also by the relevant functional electrophysiological (hERG) aspects.

There are two main reasons why the heart represents one of the major targets for the pharmaceutical industry. First, in Western countries, cardiovascular diseases (CVD) are the most common causes of death (17.6 million or 30% of the total deaths in 2005, see: http://www.who.int/cardiovascular_diseases/en/), with heart failure being one of the main aspects of CVD. Treatment of these diseases generates enormous cost for the health care system [1], illustrating the size of the market for heart-related drugs.

Second, the most common cause of drug withdrawal from the market and delays in regulatory approval for marketing is owing to adverse cardiac side effects [2]. With the appearance of small molecules acting, for example, as tyrosine kinase inhibitors, the need for relevant toxicological modeling of cardiac side effects becomes more demanding as adverse effects within this organ and myelosuppression represent the most frequent side effects. While myelosuppression can be easily analyzed by standard techniques using adult mesenchymal cells, however, relevant systems to establish cardiac toxicity are still pending.

For these reasons, assessment of the pharmacological and toxicological potential of compounds is a major issue in drug discovery, development and safety pharmacology.

Corresponding author: Bohlen, H. (heribert.bohlen@axiogenesis.com)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.06.011

In vivo cardiac models

Before a potential drug candidate can be progressed into clinical studies, the regulatory guidelines require *in vivo* studies to be performed in animal models.

Toxicological studies are mainly performed in rodents (rat, mice and rabbit) but also have to be performed in non-rodent models if the rodent models lack relevance to humans (see: http://www.emea.europa.eu/pdfs/human/swp/2836707en.pdf).

To assess the potential of drugs to delay ventricular repolarization for safety pharmacology the draft of ICH guideline S7B prescribes dogs, monkeys, swine, rabbits, ferrets and guinea pigs as suitable animal models. Smaller rodents, like adult rat and mice, are explicitly excluded owing to their a markedly different mechanism of re-polarization compared to humans (see: http://www.ich.org/LOB/media/MEDIA2192.pdf).

The advantages of *in vivo* experiments in conscious or anaesthetized animals are that effects of Class I–IV anti-arrhythmic compounds and I_f blockers, but also adverse effects of non-cardiovascular new chemical entities, can be identified readily. Moreover, all routes for drug administration can be applied [3].

Furthermore, chronically tachycardic dogs or transgenic mice have been introduced as models to study heart failure and dilated cardiomyopathy [4–6].

In vivo investigations have also been conducted to analyze drug-induced heart failure by doxorubicin in both rabbit and murine models [7,8].

The disadvantages of animal testing are that higher doses of compounds may induce side effects on other organs and, therefore, increases the intricacy of analyzing cardiac toxicity [9]. Moreover, *in vivo* testing is time-consuming, allows only low-throughput and faces, last but not least, ethical problems.

Explanted hearts and cardiac tissue

Langendorff-perfused explanted hearts of guinea pig and rabbits [10–12], as well as preparations of cardiac tissue like papillary muscle or purkinje fibres from guinea pig or dog [13], are common models in cardiac safety pharmacology and belong to the 'Gold Standard' of test batteries for the investigation of new chemical entities prior to clinical trials [3].

Explanted rat hearts have been applied to investigate aspects of injuries induced by ischemic reperfusion [14], for example apoptosis [15], or to monitor particle-induced effects on cardiac function and arrhythmias [16].

Disadvantages of these *ex vivo* models are the laborious, time consuming and costly preparation and the very low throughput capacity. Additionally, standardization of these models is difficult, owing to inter-operator differences and inter-animal variations. Furthermore, the short period of time during the experimental setup that is capable for analysis severely impedes the ability to assess long-term and chronic exposure of toxic substances.

Engineered cardiac tissues

Several attempts have been made to generate three-dimensional cardiac tissues, mainly from the aspect of replacement therapy. Recent publications have described engineered tissues from dissociated embryonic chicken or neonatal rat hearts [17] and wild type embryonic stem cell-derived murine or human cardiomyocytes [18–20].

So far, strategies to introduce engineered cardiac tissues for long-term and chronic toxicity or electrophysiological screening of pharmaceutical compounds have not yet been developed in detail [21–23].

Primary cardiomyocytes

Primary cardiomyocytes isolated from different species at different stages of development (neonatal, adult) represent established models in toxicological and physiological test systems.

The advantages of primary cells are manifold. All ion channels underlying the cardiac action potential can be measured functionally in their native cellular environment and state, and high-content physiological data can be obtained that predicts the complex interaction of ion channel activities [24].

Fetal cardiomyocytes reveal a proliferative phenotype shown by DNA synthesis and increase in cell numbers. Cytokinesis is lost around birth, but at that stage of development cardiomyocytes still display DNA synthesis (approximately until postnatal day 3) leading to binucleation typically found in adult cardiomyocytes [25].

The major disadvantage of primary cardiac cells as *in vitro* models is their low proliferative capacity. Hence, their use is limited for large screening applications. Adult cardiomyocytes, in particular, have to be prepared by a time-consuming and costly process, by enzymatic digestion in a Langendorff apparatus [26]. Moreover, isolation of adult cardiomyocytes is difficult, owing to the fact that the cells are tightly connected via gap junctions. Enzymatic digestion destroys gap junctions and leads to a permeability of the cell membrane for calcium ions and loss of electric coupling capabilities. In turn, the influx of calcium ions causes calcium overload, hypercontracture and cell death [25].

Additionally, isolated fetal and neonatal myocytes are always contaminated with other cell types (e.g. fibroblasts). These cells will start to proliferate and overgrow cardiomyocytes after a few days in culture [27] as long as they are not inhibited by cytostatic drugs or irradiation, which may interfere with reliable pharmacological or toxicological screening.

Cell lines

Several attempts to generate cardiomyocyte-like cell lines have been made to overcome the limitation of primary cell preparations. One of the first directed experiments was the transgenic expression of the viral large T-antigen under the control of the atrial natriuretic factor (ANF) promoter in the mouse, which led to the generation of the atrial tumor cell line AT-1 [28]. These cells were neither capable of serial passaging in culture, nor freezing and had to be propagated as a subcutaneous tumor lineage in syngeneic mice. A derivative of these cells, the HL-1 cell line, is capable for passaging and can be frozen and thawed from N2 stocks without loss of contractility. The properties of these cells have been well characterized as reviewed by White *et al.* [27].

In another approach, transgenic mice were produced expressing SV40 large T antigen under the control of the cardiac-specific promoter Nkx2.5 with flanking loxP sites. The mice produced cardiac tumors from which cardiomyocytes have been isolated and cultured. Adenoviral transduction with Cre-recombinase led to controlled cell cycle-arrested cells that adopted characteristics of differentiated cardiac cells [29].

A transformed cardiomyocyte cell line W1 was derived by transfection of highly enriched human fetal cardiomyocytes with the pSV2Neo and pRSVTAg constructs [30].

Isolated from spontaneously transformed embryonic rat heart, the H9c2 represents another cardiomyocyte-like cell line displaying an undifferentiated phenotype [31,32]. These cardiomyocyte cultures have been used to explore oxidative stress [33] and doxorubicin-induced cardiac cytotoxicity [34,35].

Advantages of these transformed cell lines are that they can be standardized, produced in sufficient amounts, and are capable for long-term storage as frozen stocks.

Other cell lines comprising heterologous expression of human ion channels in stable cell lines (e.g. HEK, CHO) or Xenopus laevis oocytes have been successfully generated to identify drug–ion channel interactions and are capable for the application in automated high-throughput electrophysiological analysis [36,37]. Lacking the natural cardiac cellular environment, data acquired from these cell systems have limited reliability. For example, alpha subunits of ion channels, like the hERG channel, require additional expression of the beta subunit MiRP1 to mimic the natural current [38]. As demonstrated for hERG ion channel expression, Xenopus laevis oocytes express their own interacting proteins that influence hERG currents as shown by miRNA suppression of different xenopus laevis Mink related protein (xMiRP) subunits [39]. This demonstrates that heterologous expression systems are artificial models which lack a natural cardiac enviroment.

Primary-like embryonic stem cell-derived cardiomyocytes

The first embryonic stem cell lines from mouse were described in 1981 [40,41] and generation of the well-known cell line D3 and its differentiation into cardiac myocytes was published in 1985 by Doetschman *et al.* [42]. Almost 20 years later, the generation of human ES cells and differentiation into cardiomyocytes were described [43,44].

One disadvantage of these wild type ES cell-derived cardiomyocytes is that the remaining amount of undifferentiated ES cells, as well as other proliferative cell types, will overgrow the desired cardiomyocytes within a few days. Therefore, several attempts have been made to isolate ES cell-derived cardiomyocytes, for example by percoll gradient centrifugation for tissue engineering [22] or by laser microdissection for predictive cardiotoxicity [45]. Cardiomyocytes derived from either human ESC lines or those differentiated from IPS derived stem cells have been generated, but have not yet been tested in standard assay systems.

One great advantage of murine ES cells is their susceptibility for genetic manipulation. In order to purify and identify ES cell-derived cardiomyocytes, bicistronic vectors have been used in which the cardiac-specific promoter, MHC, drives the expression of both the puromycin resistance gene and an EGFP cassette (Figure 1A) as described recently [46]. When differentiating within ES cell aggregates (embryoid bodies) the first clusters of EGFP+ cells are detected in the embryoid bodies (EBs) on days 7–8 of development (Figure 1, B1), and spontaneous beating occurs approximately 12–24 h later. Owing to the tissue-specific expression of puromycin resistance, addition of puromycin on days 9–10 results in dramatically increased EGFP fluorescence and contractile activity of the EGFP+ clusters after 24–72 h, indicating cardiomyocyte enrichment (Figure 1, B2). After 6 days of puromycin treatment, most of the EBs consist of beating EGFP+ clusters of cardiac cells [46]. Both differentiation and drug selection has been scaled by mass-culture protocol with an approximate yield of 10^8 highly purified cardiomyocytes from an initial 5×10^6 transgenic ES cells. The purity of about 99.9% has been shown by transplantation experiments in which, subsequent to transplantation of such cells in syngeneic mice, no teratocarcinoma were observed [46]. Moreover, it was shown that resulting cardiomyocytes can be frozen and thawed and retain their functional and morphological integrity as judged by both immuno-histochemical and functional analyses (Figure 1, C1 and C2) comparable to findings from cryopreserved human embryonic stem cell-derived cardiomyocytes [47]. This unique feature of the ES cell-derived cardiomyocytes allows stockpiling and distribution of the cells for purposes of functional *in vitro* models, drug and cardiotoxicity screening, cardiac electrophysiology studies, tissue engineering, and *in vivo* transplantation experiments.

A typical example of investigating the cardiotoxic effect of a complex drug is shown in Figure 2. To analyze for adverse cardiotoxic effects, test compounds are tested on ESC-derived cardiomyocytes and, in parallel, on non-cardiac reference cells, for example embryonic fibroblasts. This strategy gives the ability to distinguish between specific cardiotoxic events and general cytotoxic effects. In Figure 2, an example of a cardiotoxicity test using ESC-derived cardiomyocytes is shown. In order to demonstrate the clinical relevance of the *in vitro* system, a drug combination containing PPAR-agonists was analyzed in detail. In particular, rosiglitazone-containing drug-combinations have recently obtained attention since increases in cardiovascular risk in type 2 diabetes patients has been recently described [48,49]. In the experiments described here, rosiglitazone was dissolved from Avandia® tablets and tested at various dilutions. As compared to control fibroblasts, no cardiac-specific toxicity was observed as also described by Erdmann and Wilcox [50]. By contrast, Avandamet®, a composite drug consisting of rosiglitazone and metformin, displays a clear and specific cardiotoxic effect (see A1 and A2 in Figure 2, effect of rosiglitazone + metformin). While fibroblasts are affected only marginally, survival of cardiomyocytes is reduced substantially at higher Avandamet concentrations. Together with the data obtained with rosiglitazone as a single substance, this observation leads to the conclusion that metformin may have an adverse effect on cardiomyocytes. Therefore, metformin as a single compound was analyzed individually. As shown in Figure 2, cardiomyocyte survival is strongly reduced in the presence of metformin, strengthening the observation that the cardiotoxic effect observed with Avandamet is most likely owing to the metformin component within the formulation. The results observed in embryonic stem cell-derived cardiomyocytes support the data published by An *et al.* [51] that metformin is able to induce cardiotoxic events.

Biomarkers of cardiac damage

To expand further the descriptive capacity of ES cell-derived cardiomyocytes, biomarker release has also been studied. The term 'biomarker' was standardized by an NIH working group in 2001 as 'a characteristic that is both effectively measured and evaluated as an indicator of normal biological processes, pathogenic processes,

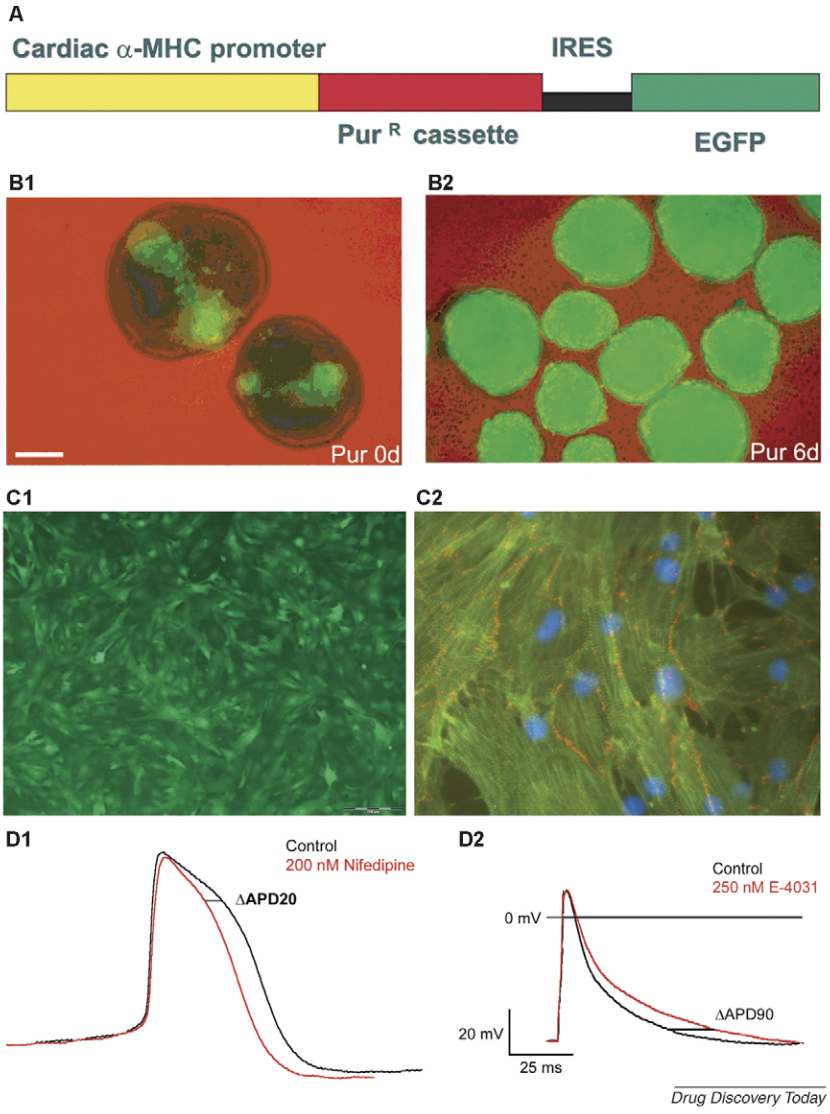

FIGURE 1

Puromycin-based selection and purification of ES cell-derived cardiomyocytes. (A) Scheme of the bicistronic vector. (B) EGFP+ areas in EBs at day 9 (B1) and efficient selection after 6 days of treatment with puromycin (10 μg/ml) (B2); (C) ES cell-derived purified cardiomyocytes were frozen, thawed, and plated on fibronectin coated plates. After 7 days, the monolayer of beating, GFP positive cardiomyocytes (C1) displayed well developed cross-striated sarcomeric structure and gap-junctions as revealed by anti Actinin (FITC) and anti-connexin 43 (Cy3) immunostaining (C2). Nuclei were stained blue with DAPI. (D) Decreasing and increasing chronotropic effects of the Ca^{2+} blocker nifedipine (D1), and E4031, the blocker of the I_{Kr} mERG, the human hERG channel analog (D2).

or pharmacological response to a therapeutic intervention' [52]. At present, there are only a few accepted biomarkers for cardiac damage that can be measured in biological samples: cardiac troponin T and cardiac troponin I as markers for myocardial necrosis; ischemia-modified albumin (IMA) as a marker for ischemia of cardiomyocytes and type B natriuretic peptides as markers for acute and chronic cardiac failure (for review, see [53]). Cardiac troponins are highly specific, stably released in the circulation [54] and the natural background level is not detectable by common assay systems. Therefore, determination of cardiac troponin was established as the 'gold standard' for detection of necrosis of cardiomyocytes [55].

In the patient, cardiac troponins can be released owing to different mechanisms of cardiac damage: myocardial infarction is the most common cause, but non-ischemic damage of cardiac tissue (e.g. inflammation, cardiac trauma, drug-induced necrosis) will also result in elevated troponin levels.

As mentioned above, ES cell-derived cardiomyocytes also allow analysis of apoptotic events. The trigger of either caspase-dependent or independent pathways will lead to DNA fragmentation (DNA-laddering), as well as conversion of AnnexinV, which can be detected in standard AnnexinV systems.

Moreover, these cells can be used to monitor electrophysiological behavior. It was clearly demonstrated by patch-clamp analysis of single cells, as well as by field potential analysis on multi-electrode arrays, that ES cell-derived cardiomyocytes express all ion channels that are present in embryonic mouse heart cells [56,57].

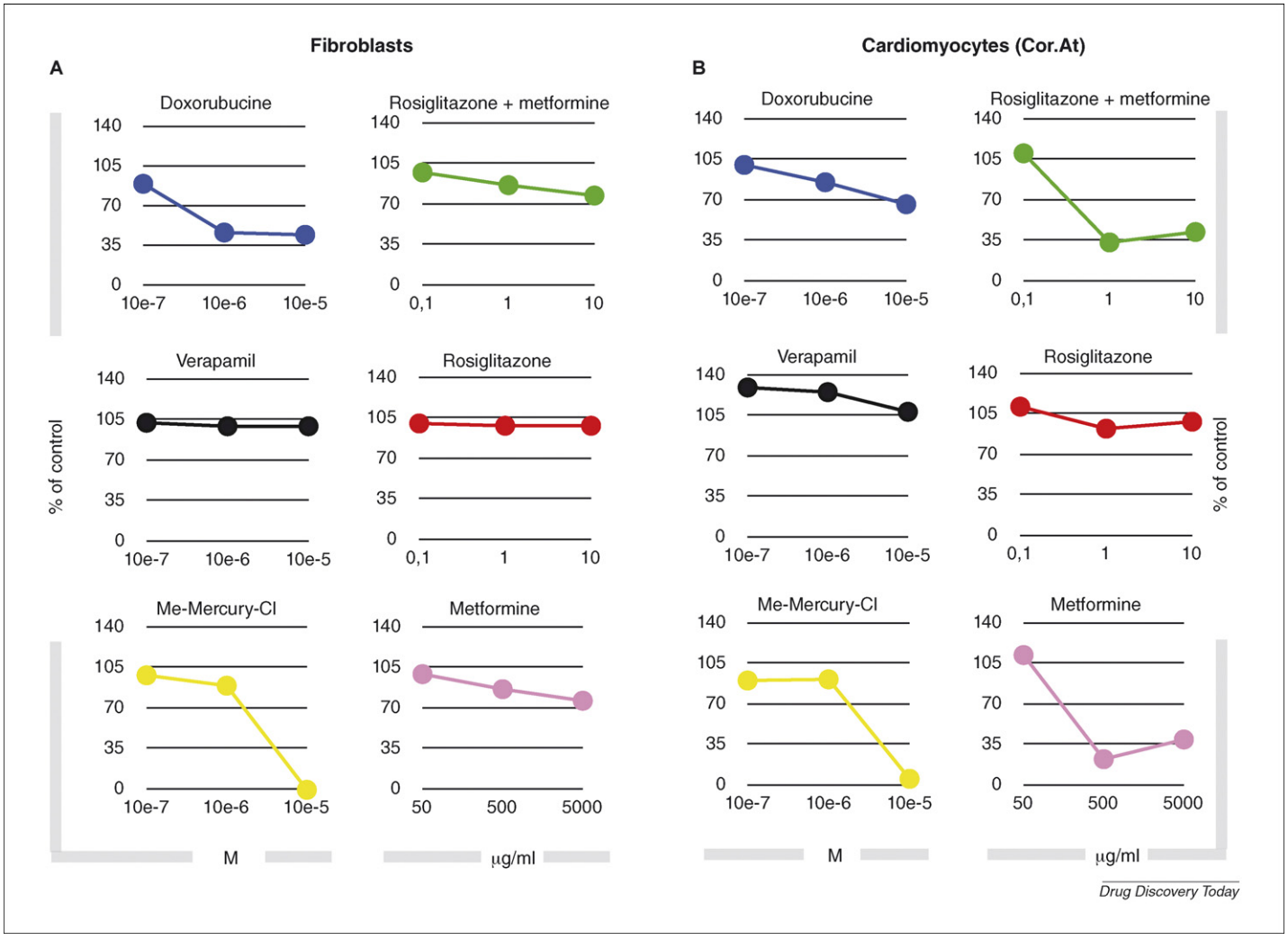

FIGURE 2

Determination of cardiac specific cytotoxicity. Embryonic fibroblasts (A) and ESC-derived cardiomyocytes (B) were seeded in 96 well plates and cultured for 48 h before the experiment. Cells were incubated with different concentrations of doxorubicin, a cardiotoxic compound, diclofenac as negative control, and methyl mercury chloride as a cytotoxic compound for 48 h (left panels). Cell survival was determined by the neutral red uptake (NRU) test relative to untreated controls. To elucidate the action of a recent clinical drawback, rosiglitazone, metformin, and a combination of both (Avandamet) were tested using the same experimental setup (right panels). Results are described in detail in the text.

Conclusion

Taken together, there is growing evidence that ES cell-derived cardiomyocytes provide a highly relevant and robust system to detect accurately the cardiotoxic potential of compounds. Since ES cell-derived cardiomyocytes can be stored as pre-seeded multiwell plates and pre-seeded coverslips, the use of such a system for medium to high throughput screening at early stages of drug development is favorable. Even more, because of its high physiological relevance and clinical predictive qualities, this ES cell-based system may be regarded as a prototype to realize toxicological modelling of substances to prevent misrouted development, especially at stages preceding clinical trials.

The use of cardiomyocytes with a disease phenotype (i.e. hypertrophic cardiomyocytes) in the above-described systems will contribute to a complete picture of the adverse cardiotoxicological capacities of a compound, even in patients with cardiac risk factors. This quality may, in the future, lead to new drug development strategies by *in vitro* modeling of patient-subgroups, thus increasing the safety- and function-profiles already in pre-clinical stages of development.

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