Cor.At® Stem Cell Derived Cardiomyocytes as a Screening System to Predict Cardiac Specific Cytotoxicity

By Silke Schwengberg
Axiogenesis AG, Cologne, Germany

Introduction

Cardiac side effects are the most common cause of drug withdrawal from the market and delays in regulatory approval of new drug candidates,[1] regardless of whether these drugs act on the cardiovascular system or not. Coherently, testing for cardiovascular safety is a major bottleneck within the drug development process, and lack of good cellular models further worsens the situation. Additionally, in Western Countries, cardiovascular diseases (CVD) themselves are the most common cause of death (17.6 million or 30% of the total deaths in 2005[2]), with heart failure being one of the main aspects of CVD. Treatment of these diseases generates enormous cost for the health care system,[3] illustrating the size of the market for heart-related drugs, and the need for suitable pharmacological models.

The most common *in vitro* models for assessment of the pharmacology and toxicity of drug candidates are (recombinant) cell lines or primary cardiomyocytes isolated from different species. However, recombinant cell lines lack the physiological properties like ion channel environment and functional humoral regulation. Although primary cardiomyocytes demonstrate *in vivo* physiological properties, they are costly, time consuming to produce, difficult to standardize, and contaminated with other cell types.[4] These models are therefore not suitable to determine whether a given compound specifically exerts a toxic effect on cardiomyocytes, but not necessarily on other cell types of the heart, e.g., fibroblasts.

During the last years, embryonic stem cells (ES cells) have been employed by researchers in the field of regenerative medicine, but also as a versatile tool to generate *in vitro* test systems. Cor.At® Cardiomyocytes are derived from transgenic mouse embryonic stem cells (mESC) harbouring a puromycin resistance cassette under control of the cardiomyocyte specific a-myosin heavy chain-(aMHC) promoter.[5] Using this approach, a population of pure Cor.At® Cardiomyocytes can be selected from the large amount of non-cardiomyocytes during differentiation of mESC. The resulting Cor.At® Cardiomyocytes are then dissociated and cryopreserved as a single cell suspension for long term storage in liquid nitrogen. Stringent quality control strategies are implemented to guarantee lot-to-lot reproducibility and uniformity of functional properties of Cor.At® Cardiomyocytes for a storage period of at least 15 months.

Thawed Cor.At® Cardiomyocytes readily form spontaneously and synchronously contracting monolayers overnight. Extensive characterization of these cells shows that they are functionally active with normal cardiac electrophysiology in terms of action potentials as well as the cardiac ion currents I_{Na}, $I_{Ca,L}$ and I_K.[6] Therefore, this model provides a homogenous and reproducible cardiomyocyte cell system, addressing the supply and reproducibility issues of primary cells while maintaining the biological significance of the data generated.

Using Cor.At® Cardiomyocytes as the specific target cell type in comparison to mouse embryonic fibroblasts (MEF) as a reference cell type, we have developed Cor.At® Tox, a robust and predictive test system to assess cardiac specific cytotoxicity, i.e., the effect on viability and integrity of cardiomyocytes. The effect of a given compound on Cor.At® Cardiomyocytes as well as on MEF is assessed using the Neutral Red Uptake test as read-out (Borenfreund E and Puerner JA 1985). This read-out system provides ease in handling as well as a high signal-to-noise ratio. With this approach, we have tested 39 anti-neoplastic compounds from different chemical classes, and compared our results with animal as well as clinical data (see Table 1). Many of the anti-neoplastic agents currently in the clinic are known for their cardiac side effects,[7] suggesting them as a good model to assess the predictivity of the assay.

Table 1

Compound List According to WHO Classification	
Alkylating Agents	Cyclophosphamide, Ifosfamide, Dacarbazin
Antibiotics	Bleomycin, Daunorubicine, Doxorubicine, Doxorubicine liposomal, Epirubicine, Idarubicine, Mitoxantrone
mAb	Bevacizumab, Cetuximab, Rituximab
Platinum-based Agents	Carboplatin, Cisplatin, Oxaliplatin
Anti-Metabolites	Cytarabin, 5-Fluorouracil, Gemcitabin, Fludarabinphosphat, Pemetrexed, Methotrexat, Pegaspargase
Plant Alkaloids	Docetaxel, Paclitaxel, Vinblastin, Vincristin, Vinorelbin, Etoposid, Etoposidphosphat
Campothecin Derivates	Irinotecan, Topotecan
Antiviral Agents	Cidofovir, Ganciclovir
Tyrosinkinase Inhibitors	Imatinib, Dasatinib, Sorafenib, Sunitinib, Lapatinib, Erlotinib

Materials and Methods

Cor.At® Cardiomyocytes were thawed and seeded at 2 x 10^4 cells per well on fibronectin-coated 96-well TC plates as described in the Technical Manual.[8] Irradiated mouse embryonic fibroblasts (MEF) were seeded at 1.5 x 10^4 cells per well on fibronectin-coated TC plates in the same medium as the Cor.At® Cells. Cells were incubated at 37°C, 5% CO_2 and 95% humidity. A complete medium change was performed 24 hours after thawing, and subsequently every 48 hours.

After 3 or 14 days of pre-culture (neonatal or adult phenotype), medium was changed and test compounds were added at different concentrations as indicated in the figures. Appropriate vehicle controls were performed for each compound on the same plate. After 48 hours of treatment, compound solutions were removed, cells washed twice with PBS, and the Neutral Red Uptake test was performed as described in the Cor.At® Tox Technical Manual.[9]

For data analysis, blank absorption values were subtracted from all other values. Mean vehicle control values were set as "100%", and values of treated cells were expressed as "% of control" in a dose response curve. All dose response curves represent the mean of at least six datapoints per compound concentration. SEM was below 10% for all compounds and concentrations. EC_{50} values were derived from the dose response curves using an automated data evaluation software. If the effect of a given compound on Cor.At® Cardiomyocytes was more pronounced than on MEF (e.g., lower EC_{50} values in Cor.At® Cardiomyocytes), the compound was classified as cardiotoxic. If the compound affects Cor.At® Cardiomyocytes and MEF in the same way, the compound was judged as generally cytotoxic.

Results

By comparing effects on Cor.At® Cells with effects on MEF, 18 out of 39 compounds were specifically toxic to ES cell derived cardiomyocytes, and 15 out of 39 were not. A review of the literature suggested 16 of the selected 39 compounds were cardiotoxic in humans (e.g., myopericarditis, ischemia, pericarditis, myocardial infarction; two false positives), 17 out of 39 were not cardiotoxic (two false negatives), and six out of 39 compounds had no data reported for cardiac specific damage in the literature, although these compounds have been on the market already for years. Thus, we identified two false negatives as well as two false positives. The six compounds lacking human clinical data were not toxic in this assay. The statistical analysis revealing an overall predictivity of 90% is summarized in Table 2.

A detailed overview of the results and clinical data can be requested at drugdiscovery@lonza.com.

Table 2

Definition (conservative):
in vivo neg. - *in vitro* unpredictable = false positive
in vivo pos. - *in vitro* unpredictable = false negative

Cardiac cytotoxic in humans	Prediction *in vitro*		Contingency tables	
	No	Yes		
			Specificity	a/a+b
No	a	b = false pos.	Sensitivity	d/c+d
Yes	c = false neg.	d	Negative predictive value	a/a+c
			Positive predictive value	d/b+d
			Accuracy	a+d/a+b+c+d

No. of compounds	Cardiac cytotoxic in humans	Prediction *in vitro*		Contingency tables in %	
		No	Yes		
				Specificity	91
23	No	21	2	Sensitivity	89
				Negative predictive value	91
16	Yes	2	14	Positive predictive value	89
				Accuracy	90

In Figure 1, representative dose-response-curves of three anthracyclins are displayed. Anthracyclins are one of the most effective classes of anti-neoplastic agents with broad spectrum anti-tumor activity. However, it was realized that these anthracyclins induced irreversible cardiac damage in patients on chronic therapy.[10]

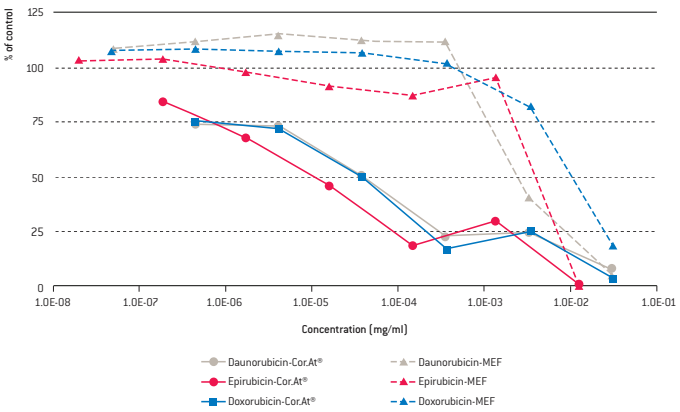

Figure 1

Effect of three anthracyclins - Doxorubicin, Daunorubicin, and Epirubicin - on viability of Cor.At® Cardiomyocytes in comparison with MEF. All three anthracyclins exhibit a pronounced toxic effect on Cor.At® Cardiomyocytes with IC_{50} values ranging from 14 - 53 ug/mL. In contrast, the effect of Daunorubicin, Doxorubicin and Epirubicin on MEF was much lower, showing a toxic effect only at the two highest concentrations tested.

Most TKI tested in the study showed enhanced toxicity on Cor.At® Cardiomyocytes compared to MEF (Figure 2). There was a strong correlation between published clinical cardiotoxicity[11] and the effect of the TKIs on Cor.At® Cells (Table 3). It is remarkable that the various TKI tested in the study displayed similar signatures, even with such a simple read-out as the NRU test.

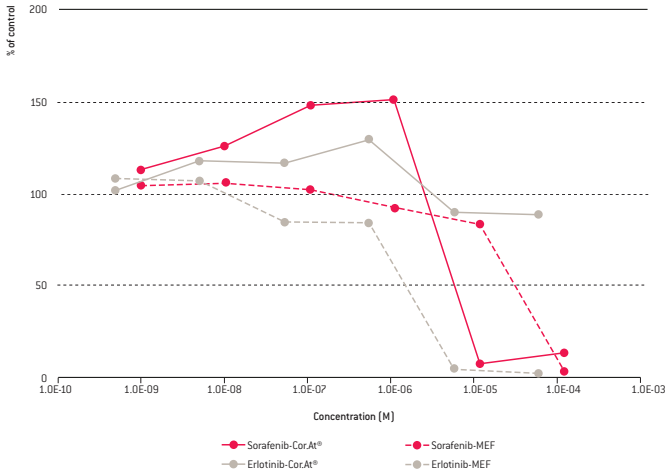

Figure 2

Effect of two tyrosin kinase inhibitors (TKI) - Sorafenib and Erlotinib. Using the quotient of IC_{50} values of Cor.At® Cardiomyocytes and MEF, Sorafenib was the most cardiotoxic TKI, and Erlotinib showed no specific cardiac toxicity at concentrations between 1 nM and 0.1 mM (see Table 3). The profile of toxicity of the TKIs on mouse ES-derived cardiomyocytes was not the same as that on MEF, suggesting that specific cardiomyocyte toxicity could be distinguished from a more generalized cytotoxic effect in these assays.

Conclusion

Our results demonstrate the reliability and specificity of Cor.At® Cardiomyocytes as a convenient model to predict cardiac specific cytotoxicity. Improving the ability to determine potential cardiotoxic effects of compounds in early stages of drug development reduces costs and improves productivity, providing better measures to move lead compounds toward preclinical and clinical development.

References

1. Gussak, I. *et al.* (2004). *J. Electrocardiol. 37, 19–24.*
2. http://www.who.int/cardiovascular_diseases/en/
3. Mensah, G.A. and Brown, D.W. (2007). *Health Aff. 26, 38–484.*
4. White, S.M. (2004). *Am. J. Physiol. Heart Circ. Physiol. 286, H823–829.*
5. Kolossov E. *et al.* (2006). *J. Exp. Med. 203. 2315–2337.*
6. Hescheler J. *et al.* (2004). *J. Electrocardiol. 37, 110–116.*
7. Jones RL and Ewer MS (2006). *Expert Rec Anticancer Ther 6(9): 1249–1269.*
8. *Cor.At® Technical Manual.*
9. *Cor.At® Tox Technical Manual.*
10. Swain S *et al.* (2003). *Cancer 97(11): 2869–2879.*
11. Orphanos GS *et al.* (2009) *Acta Oncol. 48(7): 964–70.*

Mouse ES derived Cor.At® Cardiomyocytes are not available for sale in North America. Please contact your Lonza Sales Representative for alternatives.

Table 3

	Ranking Cor.At®/MEF	IC_{50} NRU Cor.At®	IC_{50} NRU MEF	Cardiotoxicity *in vitro*	Reference cardiotoxicity *in vivo*
Sorafenib	1	0.229 µg/ml	12.6 µg/ml	++	J Clin Oncol 24(9): 1363–1369, 2006
Imatinib	2	0.442 µg/ml	18.6 µg/ml	++	Nat Med 12(8): 908–916, 2006
Dasatinib	3	0.203 µg/ml	4.54 µg/ml	++	Acta Oncol. 2009; 48(7): 964–70
Sunitinib	4	0.142 µg/ml	0.465 µg/ml	+	Lancet 370(9604): 2001–20019, 2007
Lapatinib	5	4.97 µg/ml	2.16 µg/ml	/	The Oncologist 12: 756–765, 2007
Erlotinib	6	ND	0.528 µg/ml	/	NA (only reports on skin and lung toxicity)