

Case study: “Three-dimensional multicell-type hiPSC-cardiac microtissues: a novel platform to study cardiac disease, cardiotoxicity and drug efficacy relevant to humans *in vitro*”

Cardiovascular diseases (CVDs) currently affect millions of people worldwide and represent a major cause of death globally, taking an estimated 17.9 million lives each year (www.who.int). The heart is also the major organ at risk of drug-related toxicity. Safety of pharmaceutical products is commonly compromised by severe adverse effects on the heart, which may include unexpected, potentially lethal, arrhythmia and heart failure in the case of chemotherapeutics or prolonged drug use for chronic conditions. There is thus an urgent unmet need to understand mechanisms underlying cardiac (patho)physiology to develop strategies that better predict cardiotoxic vulnerability and individual drug efficacy in developing new treatments. However, most animal models and preclinical test systems *in vitro* fail to capture human heart physiology adequately so that clinically-relevant readouts are not to hand. This results in poor predictivity of cardiac responses, limits progress in the drug development pipeline and means that regulatory authorities still require two animal species to be tested for cardiac effects before a drug can enter the market. Human induced pluripotent stem cells (hiPSCs) are widely considered by preclinical researchers as an option to replace some of these animal experiments but the regulatory authorities are not yet convinced there is sufficient proof. My aim here is to provide evidence that will contribute to build the case for replacement of one animal model (rabbits or dogs) by an *in vitro* human system. We acknowledge that it is still difficult to recapitulate complex multicellular heart conditions and the high degree of phenotypic variability observed in patients using hiPSC *in vitro*. However, this is mainly due to functional immaturity of hiPSC-derived cardiac tissues and the inability to identify cell-type specific contributions to complex disease pathogenesis or drug responses/cardiotoxicity. Contributions of the non-myocyte cell population to (both genetic and environmental) cardiac diseases such as fibrosis, arrhythmias and electrical conduction defects are becoming increasingly evident. Also, different cardiac cell types might respond differently to drugs and contribute to different aspects of cardiotoxicity. Several approaches have been proposed to induce cardiomyocyte (CM) maturation including tissue engineering, electromechanical stimulation and the addition of non-myocyte cell types in co-cultures¹. However, these systems often require specialized apparatus for tissue formation and mechanical/electrical stimulation, large numbers of cells and, next to CMs, an uncontrolled proportion of non-cardiomyocytes. Although attempts to control the input of non-cardiomyocytes have been made, these methods used primary stromal cells which are limited in supply and difficult to obtain from specific patients, thus affecting reproducibility.

I was co-first author in a paper recently published in *Cell Stem Cell*, in which these issues were addressed. We generated the three major cell types of the heart, cardiomyocytes (CMs), cardiac endothelial cells (ECs) and cardiac fibroblasts (CFs) entirely from hiPSCs, using developmental principles and combined these cells in three-dimensional (3D) structures we termed “cardiac microtissues” (MTs)². The Mummery lab has already published methods to efficiently differentiate CMs and ECs from a common cardiac mesoderm progenitor³ and how to generate epicardial cells (EPI) from hiPSCs⁴. In this recent study, the protocol was refined to differentiate cardiac specific fibroblasts (CFs) from intermediate epicardial (EPI) cells. Once differentiated, hiPSC-CMs, -ECs, and -CFs can be banked, cryopreserved and then thawed when necessary for downstream bioassays. MTs were formed from just 5000 cells by self-aggregation in controlled ratios (70% CMs, 15% ECs, and 15% CFs) that remained constant over time (Fig. 1A). Extensive characterization described in this paper demonstrated that cellular cross-talk among the three cell types included in the MTs is essential to promote hiPSC-CM maturation after 21 days in culture. Specifically, cardiac MTs showed broad maturation features at different levels:

- i) Structural: sarcomeres showed a better organization, evident as greater sarcomere alignment index and length (Fig. 1B, C). More mature features have been observed at the ultrastructural level by the presence of regular Z-lines, I-bands, H-zones and M-lines, caveolae, elongated and

- enlarged mitochondria with complex cristae as well as T-tubule-like structures, which only develop postnatally and are necessary for proper calcium handling (Fig. 1D).
- ii) Electrical: electrophysiology showed adult cardiomyocyte features including “notch” shaped (I_{to}) current in the action potential, more negative resting membrane potential and faster upstroke velocity than immature cardiomyocytes (Fig. 1E-G).
 - iii) Mechanical: contraction measurements showed increased contraction amplitude and faster contraction and relaxation velocity and acceleration (Fig. 1H). Calcium transient analysis revealed increased time to peak and near-adult decay times.
 - iv) Metabolic: oxidative phosphorylation activity and capacity were increased, as well as glycolytic respiration and capacity.

An advantage of this cardiac MT model is that it is entirely based on hiPSC-derived cells, creating the opportunity to use patient-specific models of adult-onset diseases that may have different cellular origins. This is illustrated by our experiments modelling Arrhythmogenic Cardiomyopathy (ACM) in MTs from a patient carrying a mutation in the desmosomal gene *PKP2*. Incorporating *PKP2*-mutated CFs in MTs as the only diseased cellular component was sufficient to induce arrhythmia in WT CMs (Fig. 1I, J) by reducing CX43 localization at the cell-cell junctions (Fig. 1K, L). This indicated that CFs and not just CMs contributed to ACM pathogenesis. Our results demonstrate the utility of this MT system in distinguishing cellular “culprits” from “victims” in cardiac diseases non-autonomous to CMs. This is likely also relevant to “environmental diseases”, such as fibrosis following myocardial infarction and microvascular disease leading to heart failure with preserved ejection fraction. The power of being able to create all cell types of the heart (atrial, ventricular, neurons, smooth muscle etc) from patient specific lines represent a versatile tool applicable to large-scale genetic studies (e.g. NIBSC, UK; EBiSC, EU; CIRM, US; CiRA, Japan) for studying “variants of unknown significance”, many of which will not only be expressed by CMs.

In addition, our results also showed this MT system is useful for drug screening. Cardiac MTs could capture negative and positive inotropic responses to known pharmacological compounds, verapamil and Bay K-8644, respectively. Specifically, verapamil treatment reduced contraction amplitude (Fig. 2A), as expected from the block of the L-type calcium channel. This was paralleled by decreased velocity and acceleration of both contraction and relaxation (Fig. 2B). By contrast, prolonged relaxation duration was induced by the L-type calcium channel agonist Bay K-8644 (Fig. 2C). Taken together with data from other compounds, these findings highlighted the potential of this model in screening drugs that affect different cell types in the heart compared to unicellular (and immature) monolayer cultures of hiPSC-CMs.

We used this MT model in a paper recently published on PNAS⁵ by our LUMC collaborator Prof. Jacques Neefjes, head of Cell and Chemical Biology. This study was designed to identify doxorubicin analogues that do not induce heart failure often associated with anthracycline cancer chemotherapeutics, including doxorubicin. The cardiotoxic effect of doxorubicin (Doxo) and two variants aclarubicin (Acla) and N,N-dimethyldoxorubicin (diMe-Doxo) was assessed in hiPSC-cardiac microtissues. Doxo exposure affected the contraction velocity, amplitude and duration in cardiac MTs, but these were not altered by the two analogues Acla and diMe-Doxo (Fig. 2A, B). Drug uptake in MTs was similar in all the groups, excluding the possibility that the differences were caused by different biodistribution of the drugs (Fig. 2C). Importantly, after 21 days in culture, human cardiac MTs were able to distinguish the “safe” variants and gave similar outcomes to those obtained in 3-month studies in mice.

The parallel outcomes of cardiotoxicity assessment in cardiac MTs and mice clearly demonstrate that this human system might improve and accelerate the evaluation of cardiotoxicity and reduce the requirement for testing in a second animal model.

To summarize, the critical advantages and novelty of this multi-cell type cardiac MT model are:

- i) derivation from hiPSCs of all cardiac cell types included in the MTs allows generation of cells from any genetic background, facilitating patient-specific applications;
- ii) structural, functional, electrophysiological, and metabolic maturation of hiPSC-CMs in MTs is comparable to post-natal heart and (larger) engineered heart tissue;
- iii) assays to evaluate a variety of standard functional parameters in MTs are available;
- iv) small number of cells per tissue construct (5000 cells/MT) makes MTs cost-effective (± 0.22 Euro/MT) and amenable to medium/high throughput upscaling for academia and pharma;
- v) no specific equipment/apparatus are required since MTs are scaffold-free, constructed and cultured in 96-well plates; one researcher can produce ~1500 MTs in 2 hours.

Using frozen stocks of differentiated cells circumvents the need for simultaneous workload in differentiation of the three cell types, allows quality control (QC) before freezing and reproducible large-scale production of many MTs from the same cell batches. Cryopreserved cells can be easily transferred and shared with other laboratories in both academia and pharma, enabling multidisciplinary collaborations. The simplicity and applicability of this MT model makes skill transfer particularly effective and feasible in all labs. Through protocol standardization, we have already trained external and internal research collaborators. To facilitate this further among potential end-users, the bench protocol is currently under revision for publication in Nature Protocols; I am co-first author.

Important aspects for consideration in moving this cardiac MT model to large scale discovery studies with pharma are specific endpoints for QC, reproducibility and robustness of the protocols. Table 1 reports an example of different structural (size, sarcomere length, sarcomere alignment index), contraction (inter-beat interval (IBI) and contraction duration) and single-cell electrophysiology (RMP, V_{max} , APD₉₀) parameters obtained from MTs; these data illustrate the low sample-to-sample, batch-to-batch and line-to-line variability, supporting its potential interest for biotechnology/pharmaceutical companies.

Automated handling systems and further down-scaling of cardiac MTs (2500 cells/MT) could increase throughput and facilitate testing of thousands of compounds for cardiac drug discovery, safety pharmacology and contribute to personalized medicine since based on hiPSCs. Collaborations with various companies (i.e. Ncardia, Sartorius, Miltenyi) with clear commitment in multiple aspects of the technology already exist, ensuring an outlet for direct implementation. Specifically, scaled production of cardiac MTs in a format suitable for high-throughput screening by robotics has been already put in place through an existing collaboration between Ncardia and LUMC.

In sum, controlled formation of hiPSC-derived MTs including the major cell types of the heart is poised to become the robust platform that pharmaceutical companies are looking for in pre-clinical safety and efficacy studies involving newly developed drugs or drugs “repurposed” for other disease indications. After validation and standardization, the regulatory authorities may include these tests in their guidelines much like arrhythmic prediction based on hiPSC-CMs through the Comprehensive *in vitro* ProArrhythmia Assay (CiPA) initiative is close to adoption by the FDA. Discussions have already been initiated with the European Centre for the Validation of Alternative Methods (ECVAM) and is pending with the International Council on Harmonization (ICH) for the preclinical drug pro-arrhythmia assessment. The incorporation of multiple cardiac cell types as described here, serves as an exemplar for other tissues including liver, kidney and nervous system which are all important disease and toxicity targets where either biopsies are not feasible or there is a stromal component to the diseases that cannot be captured by only including one cell type in an *in vitro* bioassay.

The case: Over the last 2 years I have played a significant role in developing the cardiac MT model and exploring its value in cardiotoxicity prediction. Prof Jacques Neefjes with support of Prof Christine Mummery has submitted a file to the Centrale Bureau voor Geneesmiddelen (CBG) for advice on whether a second animal model (in addition to the mice already tested) would be required for one of

the doxorubicin analogue we tested to be admitted for trial in patients. The request for a hearing has been accepted. We believe that this indicates the work is seriously being considered as an animal alternative for cardiotoxicity, thus leading to the possible reduction of hundreds of animals only in the Netherlands and thousands of *in vivo* experiments in Europe.

References

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2. Giacomelli E., Meraviglia V., Campostrini G. Et al., Cell Stem Cells 2020 (PMID: 32459996)
3. Giacomelli E. et al., Curr Protoc Hum Genet. 2017 (PMID: 29044469)
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Table 1. Variability table of morphological, contraction and single-cell electrophysiological parameters. The table reports quantitative parameters showing the expected sample-sample, batch-batch and line-line reproducibility. IBI: interbeat interval; RMP: resting membrane potential; APA: action potential amplitude; Vmax: maximal upstroke velocity; APD₉₀: action potential duration at 90% repolarization; SD: standard deviation; CV: coefficient of variation (calculated as SD/mean*100)

		Morphology			Contraction		Single-cell electrophysiology			
		Size	Sarcomere length	Sarcomere alignment index	IBI	Contraction duration (normalized)	RMP	APA	Vmax	APD ₉₀
Sample to sample variability	mean	458.1	1.79	0.026	3631.4	393.9	-71.0	102.4	52.8	254.9
	SD	35.8	0.06	0.004	819.3	118.1	6.7	10.7	32.3	89.2
	CV	7.7	3.10	13.538	22.1	31.8	9.4	10.5	61.5	33.8
Batch to batch variability	mean	504.4	1.78	0.027	3750.3	396.9	-70.6	101.8	57.3	225.3
	SD	15.0	0.04	0.001	1347.9	140.7	3.0	5.1	18.0	13.5
	CV	3.0	1.99	5.238	35.9	35.4	4.2	5.0	31.5	6.0
Line to line variability	mean	418.5	1.80	0.025	3331.2	393.5	-71.5	102.9	49.9	272.0
	SD	97.5	0.04	0.002	584.2	24.4	1.1	0.8	11.8	68.6
	CV	23.3	2.30	9.800	17.5	6.2	1.5	0.8	23.7	25.2

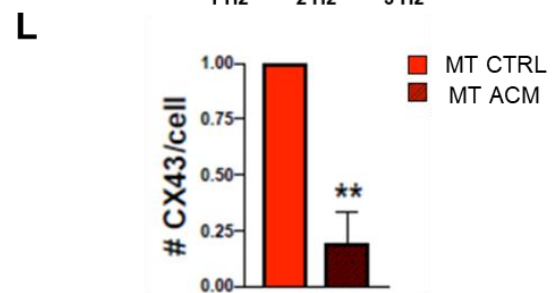
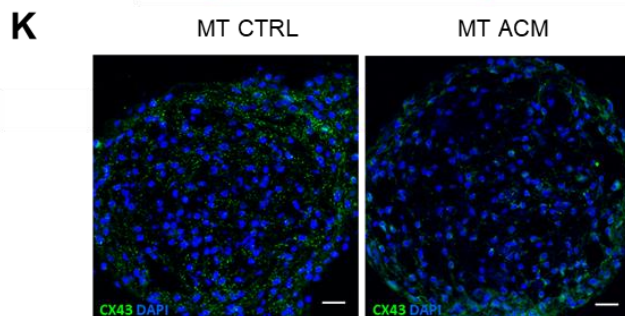
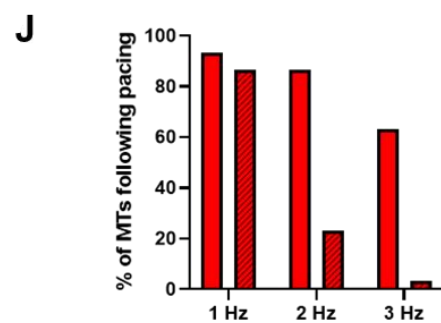
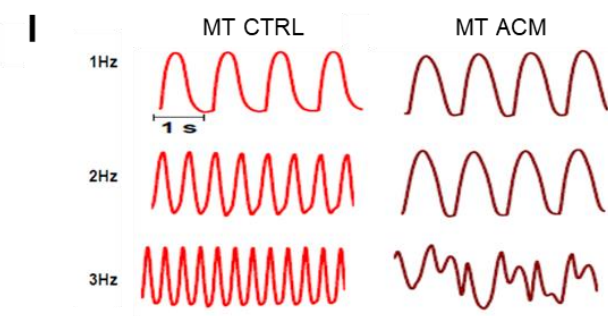
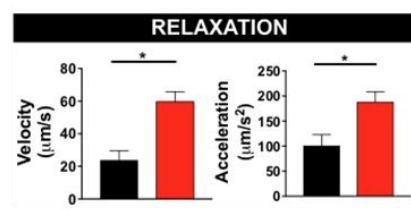
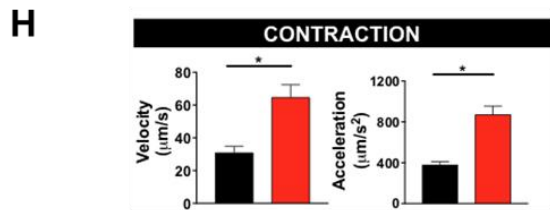
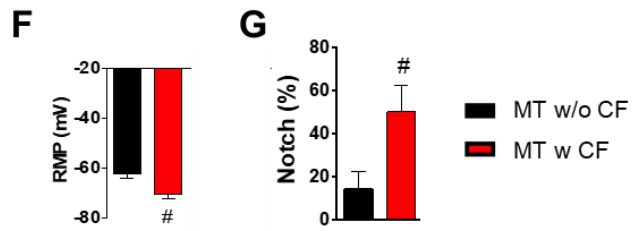
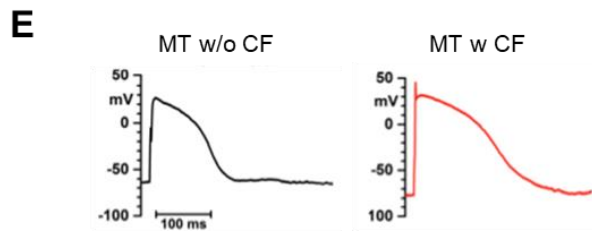
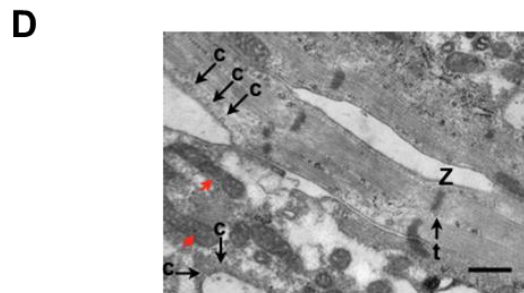
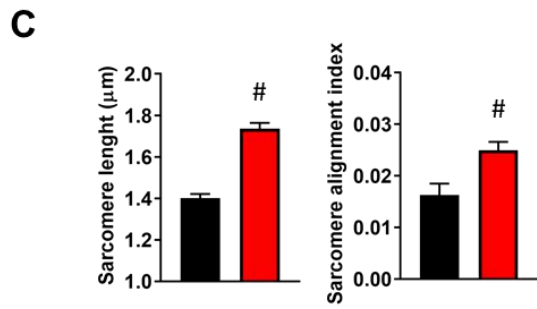
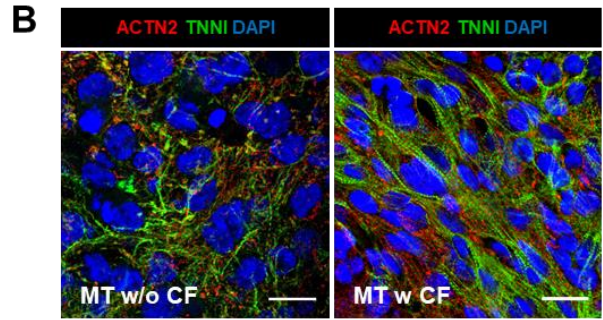
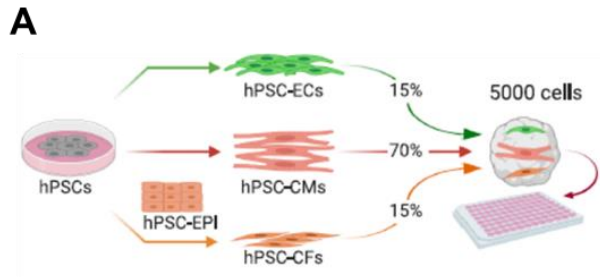


Figure 1. Three-dimensional hiPSC cardiac microtissue (MT) model and mature CM phenotype.

A) Schematic showing differentiation of hiPSCs into ECs, CMs, EPI and CFs and the combination of the three cell types into MTs. **B)** Confocal microscopy images showing improved sarcomere structure in MT with CF vs MT without CF. Scale bar: 20 μm . **C)** Sarcomere length and alignment index are higher in MT with CF. $N > 45$. t-test. $*p < 0.0001$. **D)** TEM shows presence of caveolae (c) and T-tubule-like structures (t). Scale bars: 1 μm . **E)** Representative action potential traces of hiPSC-CMs isolated from 3D-MTs without CF (black) and with CF (red). **F)** Resting membrane potential (left) is more negative in MT without CF (black) and with CF (red). **G)** A higher number of hiPSC-CMs display the “notch” shape typical of adult CMs. $N > 18$. $\#p < 0.05$. **H)** Maximum velocity and acceleration of contraction (left) and relaxation (right) is increased in spontaneously beating MT with CF (red) vs MT without CF (black). $N > 6$. t-test. $*p = 0.01$. **I)** Representative contraction traces from CTRL and ACM MTs and stimulated at 1 Hz, 2 Hz, and 3 Hz. **J)** Percentages of MTs following different stimulation frequencies in different MT groups (see legend). $N = 30$; MTs per group; Chi-square test $*p < 0.05$. **K)** Immunofluorescence analysis showing CX43 reduction (green) in ACM vs CTRL MTs. Scale bar: 25 μm . **L)** Quantification of CX43 per cell in CTRL and ACM MTs. $N = 3$; independent MT batches per group; t-test. $**p < 0.005$).

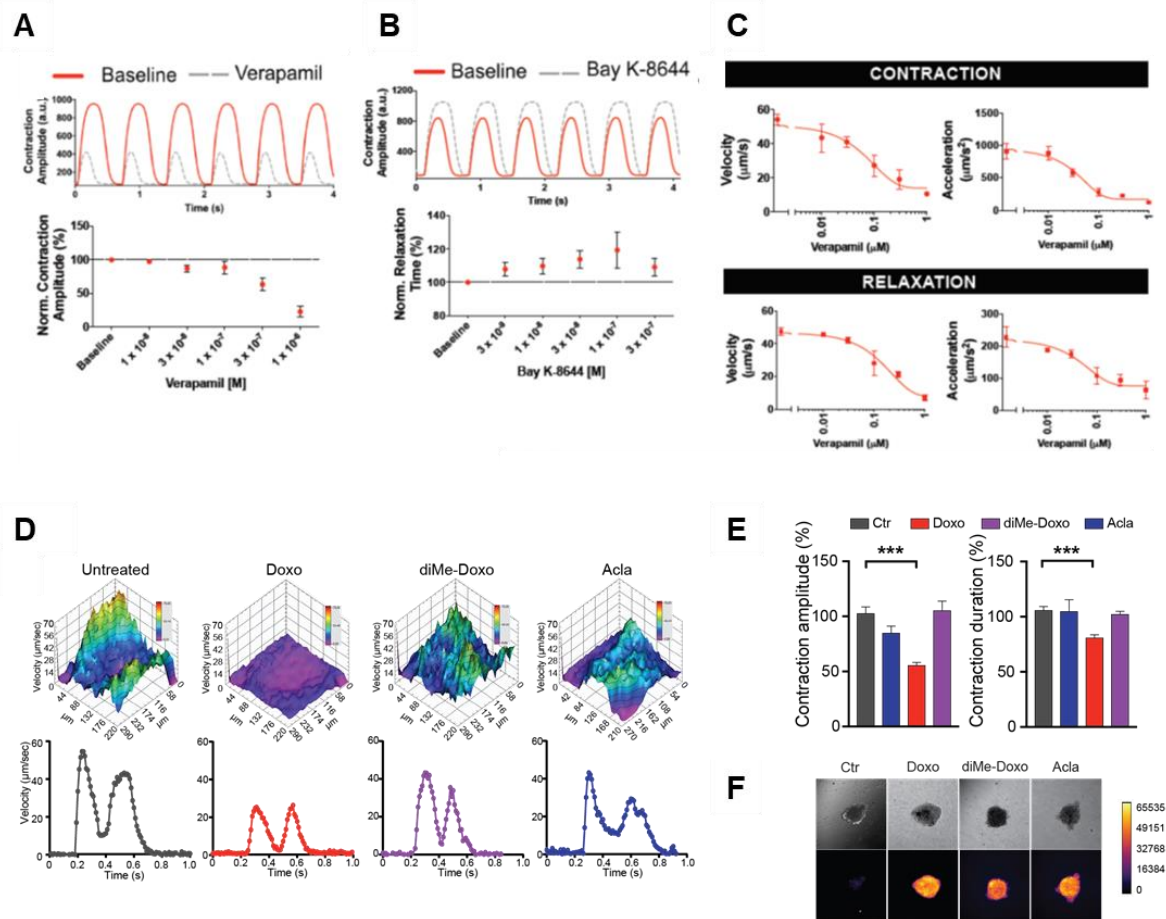


Figure 2. Cardiac MT response to pharmacological compounds and drug toxicity of anthracycline analogues in cardiac MTs treated for 24 h with 20 μM of the indicated drugs

A, B) Representative contraction traces of MT with CF under baseline condition (red lines) and upon addition of 1×10^{-6} M verapamil (**A**, top) or 1×10^{-7} M Bay K-8644 (**B**, top) (dashed grey lines) and correspondent concentration-response curves (contraction amplitude normalized to baseline) under baseline condition and upon increasing concentrations of Verapamil (**A**, bottom, N=10) or Bay K-8644 (**B**, bottom, N=6). (**C**) Concentration-response curves for velocity and acceleration of contraction (top) and relaxation (bottom) of MT with CF under baseline condition and upon increasing concentrations of Verapamil. N>14. All data are shown as mean \pm SEM.

D) Representative graphs of maximum contraction velocity for each drug treatment (top). Quantification of maximum velocity in $\mu\text{m}/\text{sec}$ (bottom). **E**) Bar graph showing contraction amplitude (left) and contraction duration (right). One-way ANOVA with Kruskal-Wallis test, ***p < 0.0002. Data are shown as mean \pm SEM. **F**) Uptake of the anthracyclines in MTs.