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Cardioprotective drugs to inhibit DOXO-induced cardiotoxicity in combination with trastuzumab for HER2-positive breast cancer treatment

Roos de Rooij
S2310864

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Bachelor Thesis Committee:
Chair: Dr. V. Schwach
Daily supervisor: Maureen Dannenberg
External member: dr.ir. J. Rouwkema

Applied Stem Cell Technologies
Faculty of Science and Technology
University of Twente

UNIVERSITY OF TWENTE.

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Cardioprotective drugs to inhibit DOXO-induced cardiotoxicity in combination with trastuzumab for HER2-positive breast cancer treatment

R.N. de Rooij

ABSTRACT

Drug treatments, like anthracyclines with Trastuzumab for Her2-positive breast cancer, face withdrawal of promising treatments caused by the major side effect, cardiotoxicity. A 3D platform of μ-engineered heart tissues (3D-μEHTs) has created the possibilityof receiving more insights into the mechanisms of cardiotoxicity. The 3D-μEHTs platform has been used for our research to investigate the contractility and sarcomere alteration of the existing anthracycline Doxorubicin and the antibody Trastuzumab. Our research has shown that Trastuzumab on itself was not cardiotoxic, even at a very high concentration. The combination of Trastuzumab with Doxorubicin, however, affects the contractility after one dose in only two days (D2, 1 μM DMSO Force of Contraction: 96,65 ± 1,38 μN, n=2; 1 μM DOXO and Trastuzumab Force of Contraction: 40,23 ± 3,75 μN, n=4). A more hopeful outcome are the signs of recovery of contractility at the end of the experiment. In addition, we performed a first *in vitro* experiment with a new cardioprotective drug which indicates no contractility protections in the 3D-μEHTs platform yet. This *in vitro* experiment has gathered information about existing and developing drug treatments for breast cancer, which affects millions of cancer patients who undergo treatment each year.

Keywords: Cardiotoxicity, anthracyclines, anti-cancer drugs, Doxorubicin, Trastuzumab,HER2-positive breast cancer, ErbB2-signalling, cardioprotective drugs, DMSO, Sarcomeres

INTRODUCTION

Breast cancer is the most frequent cancer affecting women worldwide. (1) Due to improved treatment with anthracyclines and Trastuzumab of patients with HER2-positive breast cancer, the outcome of curing HER2-breast cancer has become higher. (2) However, cardiotoxicity became an emerging problem. (3) Clinical outcomes show reduced left ventricular ejection fraction (LVEF), left atrium dilatation and heart failure. (4, 5, 6)
The human epidermal growth factor receptor-2 (HER-2) is overexpressed in 20 to 25% of human breast cancers. Trastuzumab is a humanized monoclonal antibody (mAb) directed against HER-2. (7) The drug blocks HER-2 signalling by multiple mechanisms, including disruption of the erythroblastic leukaemiaviral oncogene homolog 2 (ErbB2) signal transduction. HER-2 molecules are physically disabled and then form dimers or the next members of the HER/ErbB2 family. (7)
This is where cardiotoxicity becomes relevant. The interference of Trastuzumab with ErbB2 signalling, as the activity of both HER-3 and HER-4 is impaired when HER-2 is not available for formation ofheterodimers. (7) This signifies the cellular defensive and energy-generating systems of cardiomyocytes which are not able to function properly. (7)
This ErbB2 signalling pathway in cardiomyocytes is the protecting mechanism for anthracycline-induced apoptosis and cardiomyopathy. The anthracycline doxorubicin (DOXO) increases the production of reactive oxygen and nitrogen species. DOXO-derived oxidative or nitrative stress causes several cellularprocesses; cellular hypertrophy, extracellular matrix (ECM) remodelling due to the involvement of p38MAPK and NADPH oxidase, alterations of cardiac contraction and even cardiomyocyte death throughcytochrome c release and caspase-3 activation. The reduction of contractile function is caused by the downregulation of the activity of GATA4, a transcription factor critical for regulation of myofibrillar differentiation in cardiomyocytes. The HER-2 (ErbB2) signalling normally activates certain survival pathways which intensify oxidative and nitrative stress. (7,8) Yet, Trastuzumab will disturb ErbB2 signalling and hence the survival mechanism in DOXO treatment, which results in oxidative and nitrative stress. Due to this, the combination of DOXO and Trastuzumab leads to an increased risk of cardiac toxicity. (2)
The risk of cardiotoxicity with Trastuzumab has been reported to be 4% with monotherapy and 27% when administered in combination with an anthracycline. (9) These outcomes have given empirical datathat disrupting ErbB2 signalling in cardiomyocytes during or immediately after anthracycline treatment worsens cardiac function in patients. (7) Nonetheless, because the combination of anthracyclines with trastuzumab has shown a significantly higher cure rate than anthracycline themselves (50% versus 32%; $P < 0.001$)(2, 10), there have been ongoing experiments to analyze this in combination with cardiac protective drugs (CPDs). These CPDs can be based on Angiotensin-converting enzyme inhibitors (ACEi), Angiotensin II receptor blockers (ARBs),Beta-blockers (BB), lipid-lowering drugs, antiplatelets or even

polypills (a combination of the mentioned CPDs mechanisms). (11) Records have shown that beta-blockers contain a modest protection of cardiac function, especially in LVEF. (12) Currently, the only approved cardiac protective agent for DOXO-induced cardiotoxicity is called dexrazoxane. (13) Accordingly, there is a pressing need for experimental studies on cardioprotective agents in combination with anthracyclines and trastuzumab treatment. In this research, the side effects of Trastuzumab and Doxorubicin on contractility will be determined with a 3D µ-engineered heart tissue (3D-µEHT) platform and the performance of a new cardioprotective drug, which can be a cornerstone to anthracycline and Trastuzumab breast cancer treatment.

MATERIALS AND METHODS

Constructing organ-on-a-chip

The hPSC-cardiomyocytes will be seeded in a chip to generate the µ-engineered heart tissues. The organ-on-a-chip gel made out of curing agent and PDMS with the proportion 1:10, was poured over the organchip mold. Next, to eliminate the air bubbles, it was kept under a vacuum pressure of 0,1 bar for about at least 3x 15 minutes. After the gel was cleared of air bubbles, the gel was solidified overnight (O/N) in the incubator at 65°C. Meanwhile, glass slides were covered with PDMS and kept O/N in the incubator at 65°C to solidify. Finally, the gel chips were attached to the PDMS glass slide by utilizing ultraviolet (UV) light.

Seeding µ-engineered heart tissues (µEHTs)

Thaw cardiomyocytes (CM) of the WTC cell line were centrifuged for 3 minutes followed by resuspension in the CM medium containing glucose (5mM), lactate (4.5mM), TDI and FGF (5ng/mL). The same steps were followed for the fibroblasts (FB) of the primary human cardiac FB cell line (hacFB p11 600k). Meanwhile, the chips were coated with 1% Pluronic F127 for at least 20 min at RT until aspiration.
After this, the gel-cell suspension was made consisting of 10% fibrinogen (FN), 10% Matrigel (MAT), 1% aprotonin (AP) and 80% cell mixture (90% CMs and 10% FBs).
Finally, the chips were seeded with the gel-cell suspension combined with diluted thrombin (1:1 with 0,1% BSA) and polymerized in the chambers for 10 minutes. CF-medium was added to the chambers of the chips and stored in the incubator at 37°C and 10% CO2.
For the second experiment, the same steps were taken for the CM (D20) and FB (hacFB p11 600k) cell lines. In the end, the chips were again seeded with the same components gel-cellsuspension as before and stored in the incubator at 37°C and 10% CO2.

Contraction parameters recording in µEHTs

µEHTs in the chips were generated as described above and cultured in CM medium, refreshed each day. Contraction recording was performed 10-12 days after tissue formation and everyday refreshing at 37°C and 10% CO2 on a Nikon A1 Microscope with a 2x objective for 3 chips. The µEHTs were paced at 1,5 Hz during the measurement. After, the first measurement they were treated with drugs, 1 µM DMSO, 1 µM DOXO:Trastuzumab and 25 µM Trastuzumab. After 24 hours, a second contraction measurement was performed and refreshed with CM medium. The contraction recordings of the treated µEHTs were measured every 24 hours for 72 hours (3 days) and on days 7 and 10. Posterior to every measurement the chips were refreshed with CM medium to keep them in a convenient environment. Finally, on day 10 the last contraction recordings were generated, the cells were fixed and storedat 4°C. For the second experiment, the same measurements were executed up to D7, but for the followingdrug treatments; 1 µM DOXO, 1 µM DOXO + 10 µM CPD or 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD.

Treated µ-engineered heart tissues analysed

The videos were made with the Nikon A1 Microscope as described above and were analyzed with the HAARTA software, designed by dr.ir. J.M. Rivera Arbelaez. (14)

Immunostaining

Posterior to the recordings of the treated µ-EHTs with the Nikon A1 Microscope, they are fixated in a 96-well culture plate. This was done with 4% formaldehyde for 15 minutes and stored at 4°C until immunostaining was performed. For this, the tissues were blocked withblocking buffer; 3% BSA, 0,3% Triton, 0,1% Tween and PBS O/N. Next, primary antibodies, Troponin C (rabbit) (cTnT) (Proteintech, 15513-1-AP, 700 µg/ml) with a concentration of 1:250 and α-actinin (mouse) (Sigmaaldrich, A7811, 2ml) with a concentration of 1:800 were added to the antibody buffer solution; 0,1% BSA, 0,3% Triton, 0,1 % Tween and PBS. The wells plate was kept in the dark at 4°C for 48 hours and was made sure to keep moving. After, washing the µ-tissuesfor 3x 20 minutes with the permeabilization buffer; 0,3% Triton and PBS, the secondary antibodies were added. The secondary antibodies Donkey anti-Mouse: 647 (Invitrogen by Thermo Fisher, A31571,2mg/mL) and Chicken anti-Rabbit: 488 (Life technologies, A21441, 2 mg/mL) both with a

concentration of 1:500 were mixed with the antibody buffer solution and again the µ-tissues were stored in the dark at 4°C while moving overnight on the rocker.Finally, the secondary antibody solution was washed off with 3x PBS for 20 minutes and the DAPI staining with a concentration of 1:3000 was performed. The µ-tissues were stored in the dark at 4°C until immunofluorescence images were made.

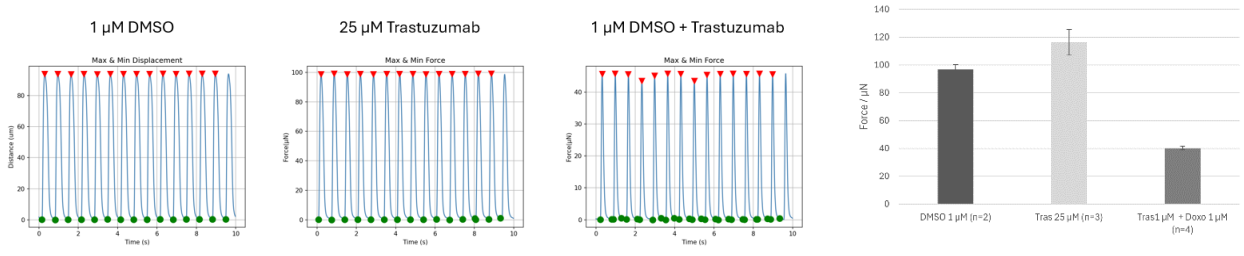
Immunofluorescence imaging

In the final analysis, immunofluorescence images were made 24 days after drug treatment with the ZeissLSM880 confocal microscope for NKX20. The settings were based on the negative control DMSO andapplied to the other conditions. In the end, the images were analysed with the software ImageJ-win64. (15, 16)

RESULTS

The combination Trastuzumab and DOXO affect contractility

Since oxidative and nitrative stress causes contractility reduction due to myofibrillar dysregulation, we proceeded to assess how several drug conditions affected the contractility of cardiomyocytes using a three-dimensional µ-engineered heart tissue (3D-µEHT) format. µ-EHTs were exposed to 1 µM DMSO, 25 µM Trastuzumab or 1:1 µM DOXO:Trastuzumab. The high Trastuzumab concentration of 25 µM was chosen to see if this extent of drug concentration will result in cardiotoxicity, like *in vivo* has already shown in clinical trials. We quantified force of contraction (FoC), contraction velocity (VEL) and relaxation VEL for D0, D1, D2, D3, D7 and D10 for an variating amount of tissues (n). After the measurement on D0, the EHTs were treated once with the drug conditions to determine possible recovery overtime. As a control for functioning EHTs, we watched them at a microscopic level to show their intact integrity which remained throughout the experiment. (Figure 1C). 24 hours after drug treatment, no cardiotoxic effects were observed. (D1, DMSO FoC: 97,96 ± 3,54%, Concentration VEL: 98,06 ± 4,20%, Relaxation VEL: 96,53 ± 3,33%, n = 2; Trastuzumab FoC: 92,26 ± 3,18%, Concentration VEL: 88,44 ± 4,26%, Relaxation VEL: 92,30 ± 2,88%, n = 3; Trastuzumab + DOXO FoC: 92 ± 1,82%, Concentration VEL: 92 2,75%, Relaxation VEL: 92,75 ± 2,15%, n = 4) (Figure 1D, E). However, proceeding to 48 hours, an extensive decrease of Force (µN) has been observed for the combination of DOXO and Trastuzumab. (1 µM DMSO Force: 96,65 ± 1,38 µN, n=2; 1 µM DOXO:Trastuzumab Force: 40,23 ± 3,75 µN, n=4) (Figure 1A, B) At D3, this toxic behaviour increased drastically which resulted in tissues which were not sensitive to pacing anymore. Therefore, the FoC together with the contraction kinetics were set at 0. (D3, Trastuzumab + DOXO FoC: 0 ± 0,46%, Contraction VEL: 0 ± 0,40%, Relaxation VEL: 0 ± 0,96%, n = 4) (Figure 1D). Notable, the high concentration of Trastuzumab however has still not shown any signs of cardiotoxicity. (D3, 25 µM Trastuzumab FoC: 103 ± 0,26%, Contraction VEL: 93 ± 0,57%, Relaxation VEL: 105 ± 0,32%, n = 3) (Figure 1D) It even shows similar behavior to DMSO from D0 up to D7, a nicely fluctuation around the normalization. (D7, Trastuzumab FoC: 108 ± 1,62 %, Contraction VEL: 86 ± 32,12%, Relaxation VEL: 112 ± 0,55%, n = 3; DMSO FoC: 112 ± 13,19%, Concentration VEL: 108 ± 9,18%, Relaxation VEL: 117 ± 13,21%, n = 2) (Figure 1D) .



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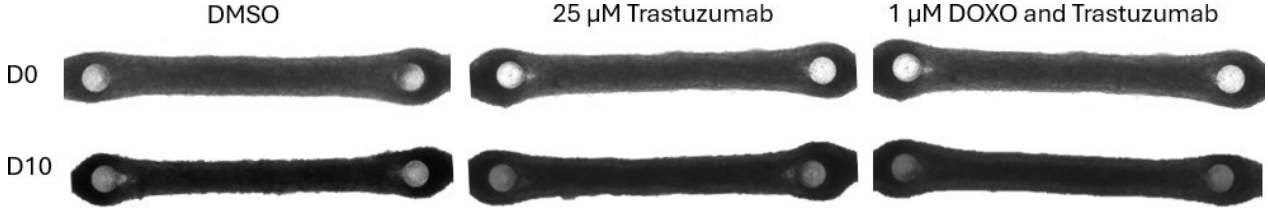

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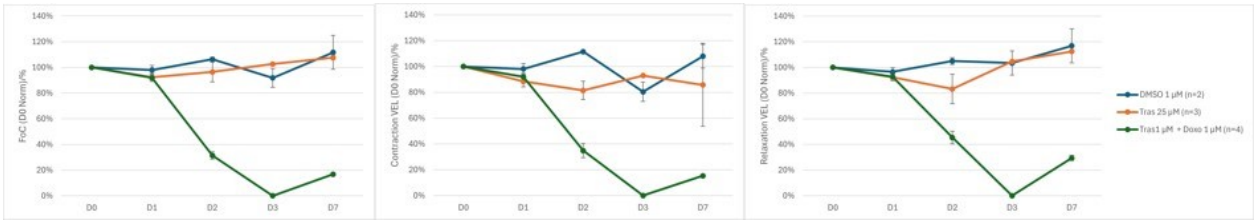

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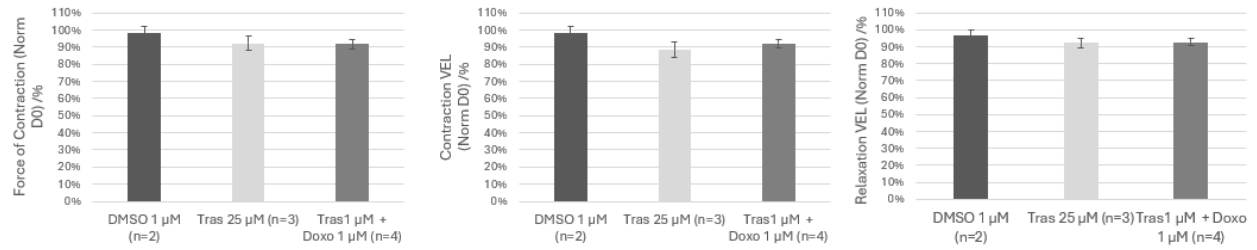


E

Figure 1. Cardiac function based on FoC and contraction kinetics
A) Representative force curve graphs.
B) Quantification of the force in µEHTs 48 h after different drug treatments. Data plotted as means with 1 µM DMSO (n = 3), 25 µM Trastuzumab (n = 3), 1:1 µM DOXO:Trastuzumab (n = 4)
C) Example micrographs of EHTs treated with 1 µM DMSO, or 1 µM DOXO:Trastuzumab or 25 µM trastuzumab at the start (D0) and end (D10) of the experiment.
D) Quantification of Force of Contraction (FoC), contraction velocity (VEL) and relaxationVEL for several days from D0 to D7 for different drug conditions after exposure. Data wasnormalized to day 0 of the experiment. (n = 2-4 EHTs per condition, from the cardiomyocytes-fibroblasts (CF) cell culture.)
E) Quantification of FoC, contraction VEL and relaxation VEL 1 day after drug exposure. Data was normalized to timepoint D0 of the experiment.

Interestingly, at D7 we discovered a recovery of contractility in the DOXO: Trastuzumab treated µ-EHTs. (D7, 1 µM DOXO:Trastuzumab FoC: 17 ± 1,34%, Contraction VEL: 15 ± 1,09%, Relaxation VEL: 29 ± 2,01%, n = 4) (Figure 1D).
At D10 an increasement of contractility seemed to continue. (D10, DOXO:Trastuzumab FoC: 22,52 ± 1,57%, Contraction VEL: 21,35 ± 2,83%, Relaxation VEL: 34,57 ± 5,80%, n = 4;) (Figure 2B) Nonetheless, an increasement in the DMSO occurred as well. (D10, DMSO FoC: 123,27 ± 7,12%, Concentration VEL: 173,25 ± 7,39%, Relaxation VEL: 214,06 ± 13,29%, n = 2) It appeared that at D10 they did not follow the pacing frequency of 1,5 Hz. (Figure 2A) Normalizing DOXO:Trastuzumab to the high DMSO values, has resulted in a significantly lower rate of recovery which makes the recovery of D10 misleading and not analyzable to the prior days. (D10, DOXO:Trastuzumab FoC: 18,27 ± 1,57%, Contraction VEL: 12,32 ± 2,83%, Relaxation VEL: 16,15 ± 5,80%) (Figure 2C)

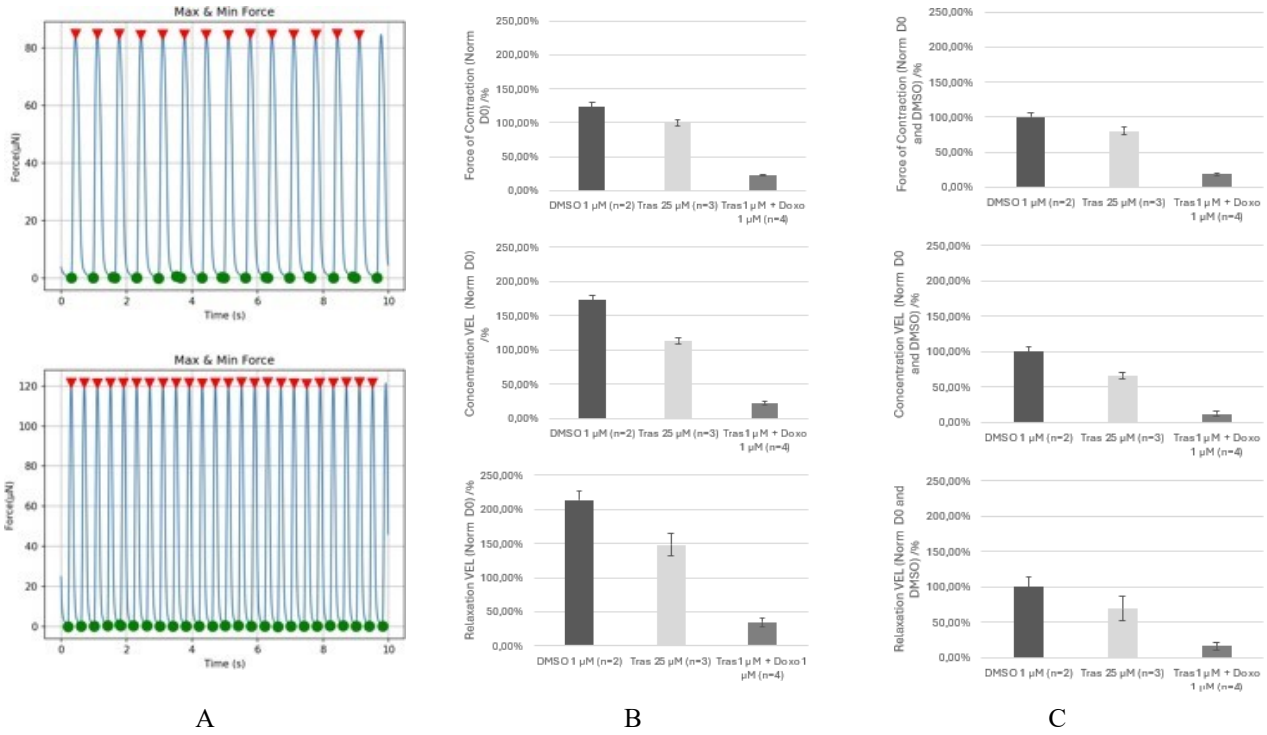


Figure 2. Cardiac function based on FoC and contraction kinetics for D10
A) The expected frequency of 1,5 Hz versus the unexpected frequency of 2,5 Hz for the negative control DMSO
B) Quantification of FoC, contraction VEL and relaxation VEL 10 days after drug exposure. Data was normalised to timepoint D0 of the experiment.
C) Quantification of FoC, contraction VEL and relaxation VEL 10 days after drug exposure. Data was normalised to timepoint D0 and DMSO of the experiment.

The cardioprotective drug affects contractility

As pronounced before, there is a pressing need for experimental studies on cardioprotective agents.After, confirming that the combination of anthracyclines with Trastuzumab was very toxic in the previous experiment, we now seek to observe the effects of a CPD. Due to some technical issues, it has only given us a small indication.
The same 3D-EHT format was used. Yet, this time the µ-EHTs were exposed to 1 µM DOXO, 1 µM DOXO + 10 µM CPD or 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD. The DOXO provides us with a positive control in addition to the negative DMSO control. For two other chips left, we used two conditions for testing the CPD, which includes the combination of anthracyclines with Trastuzumab.
As yet, this concentration of 10 µM CPD does not seem to protect the contractility as the CPD conditions are reduced to less than 50% of the original FoC. (D2, 1 µM DOXO FoC: 29,86%, Contraction VEL: 17,23%, n = 1; 1 µM DOXO + 10 µM CPD FoC: 14,40%, Contraction VEL: 23,03%,n = 1; 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD FoC: 32,02%, Contraction VEL: 48,29%, n = 1).(Figure 3B) We quantified FoC and contraction VEL for D0, D1, D2, D3 and after the measurement at D7 we decided to fixate them after no signs of survival. (D7, 1 µM DOXO FoC: 0%, Contraction VEL:0%, n=1; 1 µM DOXO + 10 µM CPD FoC: 0%, Contraction VEL: 0%, n=1; 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD FoC: 0%, Contraction VEL: 0%, n=1). (Figure 3B)
As a control for functioning EHTs, we watched them at a microscopic level to show their integrity. (Figure 3A) However, this time we noticed that already at D0 the 1 µM DOXO and 1 µM DOXO + 10 µM CPD tissue are thinner compared to the third condition and the conditions of the first experiment. At D7, they show rough edges and for the 1 µM DOXO and 10 µM CPD condition, the right pillar seems to be attached to the glass slide.

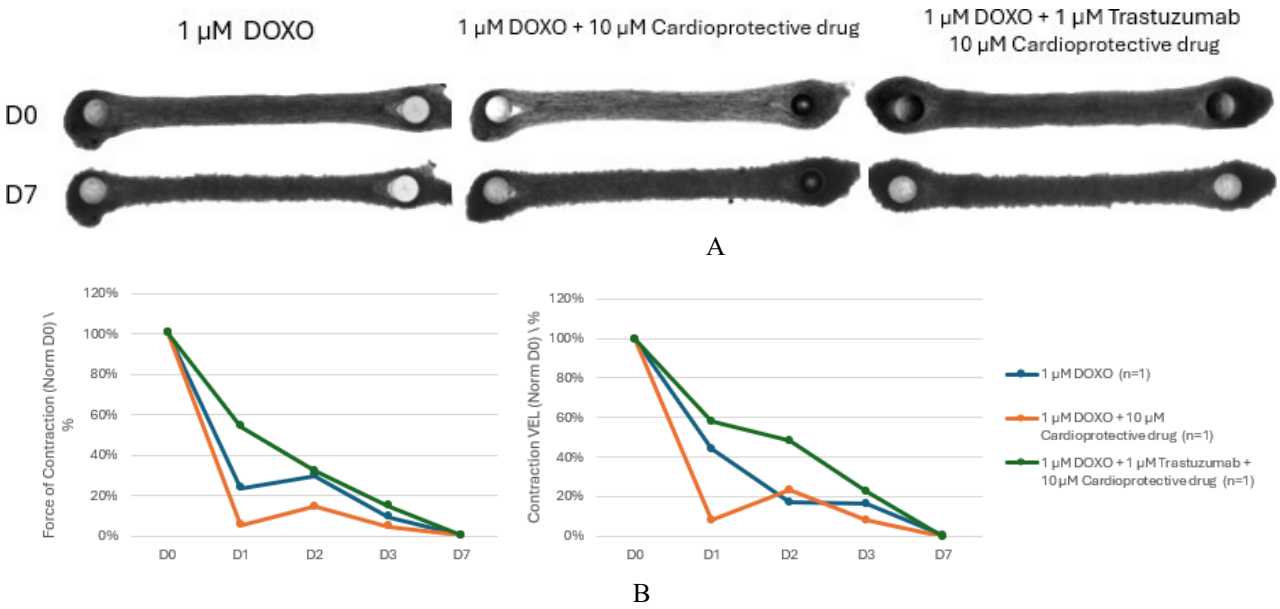


Figure 3. A) Example micrographs of EHTs treated with 1 µM DOXO, or 1 µM DOXO and 10 µM CPD or 1 µM DOXO and 1 µM Trastuzumab and 10 µM CPD at the start (D0) and end (D7) of the experiment.
B) Quantification of FoC and Contraction VEL for several days from D0 to D7 for different drug conditions after exposure. Data was normalized to day 0 of the experiment. (n = 1 EHT per condition, from the cardiomyocyte-fibroblast cell culture.)

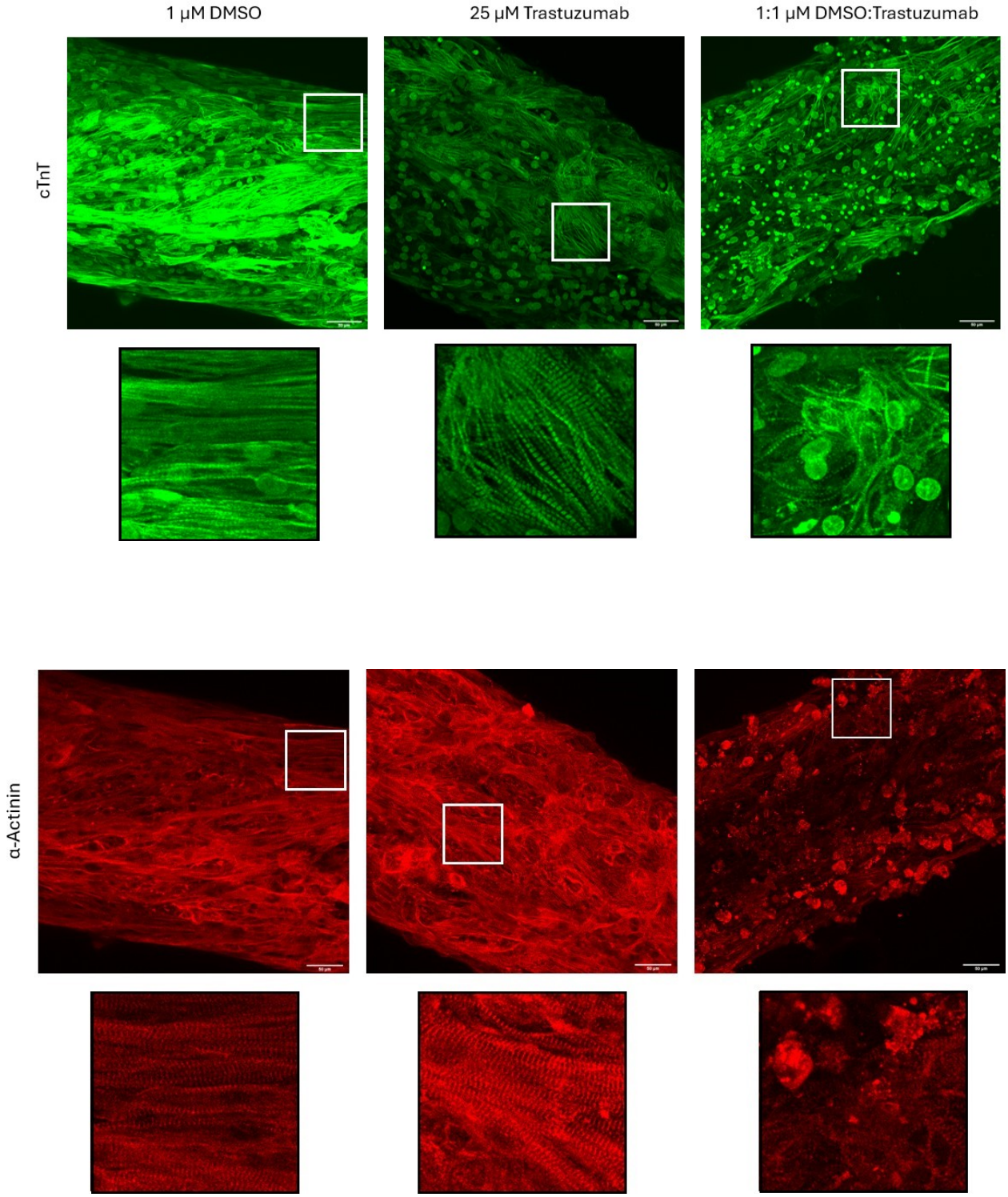
In comparison with the previous experiment, it is interesting that this time in the first 24 hours the drug treatments extensively affected the contractility. (D1, 1 µM DOXO FoC: 23,42%, Contraction VEL: 44,33%, n = 1; 1 µM DOXO + 10 µM CPD FoC: 5,07%, Contraction VEL: 7,75%, n = 1; 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD FoC: 53,91%, Contraction VEL: 57,96%, n=1) (Figure 3B).Remarkably, the DOXO condition with and without the CPD performs a quite similar pattern through the days. However, the one with the CPD at a lower level of contractility values. The most interesting is the fact that the combination of DOXO with Trastuzumab in the first experiment exposed to be the most toxic,this time appears to be the least toxic. This is the case, for each measured day and even for D3 where there was no contractility without the CPD. (D3, 1 µM DOXO FoC: 9,52%, Contraction VEL: 16,33%, n=1; 1 µM DOXO + 10 µM CPD FoC: 4,51%, Contraction VEL: 8,12%, n=1; 1 µM DOXO + 1 µM Trastuzumab + 10 µM CPD FoC: 14,67%, Contraction VEL: 22,73%, n=1) (Figure 3B)

Sarcomere organization of cardiomyocytes

The FoC and contraction kinetics have shown cardiotoxicity for the 1:1 µM DOXO:Trastuzumab. Sarcomeres play a significant role in the contraction of the cardiomyocytes. We, therefore, wanted to quantify if µEHTs suffer from cardiotoxicity, the sarcomere organization should also be affected and confirm the outcomes of the contractility analysis.

First, the immunostaining images of the control DMSO show a model of how the sarcomeres should beorganized; aligned in a parallel fashion, showcasing a transverse striation. (Figure 4) Just like the results of the FoC and contraction kinetics, the 25 µM Trastuzumab shows similar figures to DMSO. (Figure 4) As expected, the tissues are affected by the DOXO with Trastuzumab condition and therefore show immunostaining results with less intensity. (Figure 4) The immunofluorescence intensity is notably diminished compared to the DMSO control, especially for the α-actinin staining (DMSO cTnT Total Sarcomere Fluorescence (TSF): 13541985 au, *α*-actinin TSF: 2414928 au; DOXO:Trastuzumab cTnT TSF: 8170833 au, *α*-actinin TSF: 8588886 au; Trastuzumab cTnT TSF: 17325254 au, *α*-actinin TSF: 25676570 au) (Figure 5). The staining of cTnT and α-actinin in the nuclei means that a fraction of the nuclear proteins are not completely released, which is the case in all the conditions of cTnT and in DOXO:Trastuzumab of *α*-actinin. Furthermore, upon examination of the DAPI staining, a lower amount of nuclei and thus cell death is observed. (1 µM DMSO Nuclei Count: 3331, n=1; 25 µM Trastuzumab Nuclei Count: 2127, n=1; 1 µM DOXO:Trastuzumab Nuclei Count: 1617, n=1) (Figure 6) This is consistent for the sarcomeres in the *α*-actinin and cTnT staining. Besides the lower intensity, the alignment has also been disturbed compared to DMSO. Having a closer look in the comparison of *α*-actinin and cTnT staining in the shown cardiotoxic 1:1 µM DOXO:Trastuzumab condition, it shows an remarkably difference. Although, in the cTnT staining one can discern there is still some transverse striation

in the present fibrils, the aligned organization is virtually inconsistent. The *α*-actinin staining, on the other hand, has lost its organization in every way, not aligned or parallel and no transverse striation. (Figure 4)



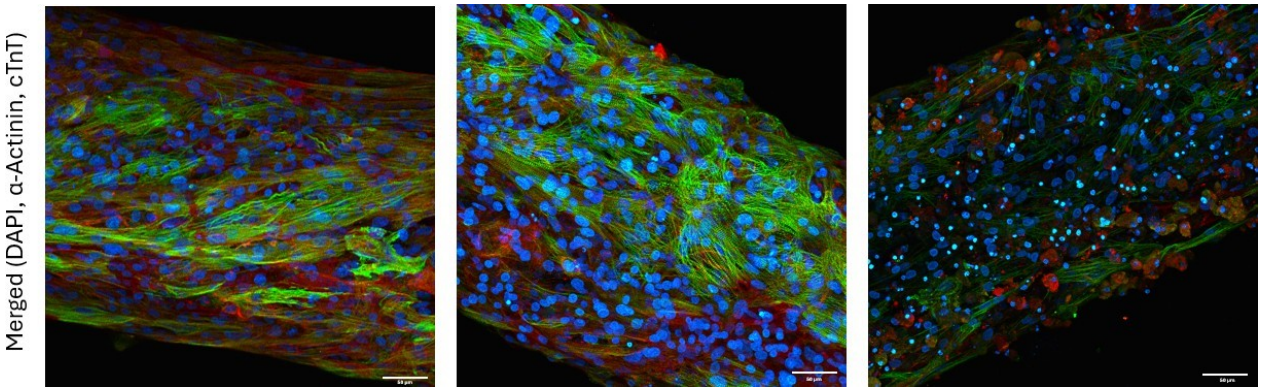


Figure 4. Immunofluorescence staining of sarcomeric *α*-actinin (red) and cardiac troponin (cTnT) (green) for treated µ-EHTs with DMSO, 25 µM Trastuzumab or 1:1 µM DOXO:Trastuzumab. DNA is stained with DAPI (blue). The different stainings are shown by themselves and merged in one image. The images have a magnification of 20x and a square close-up. Scale bar = 50µM

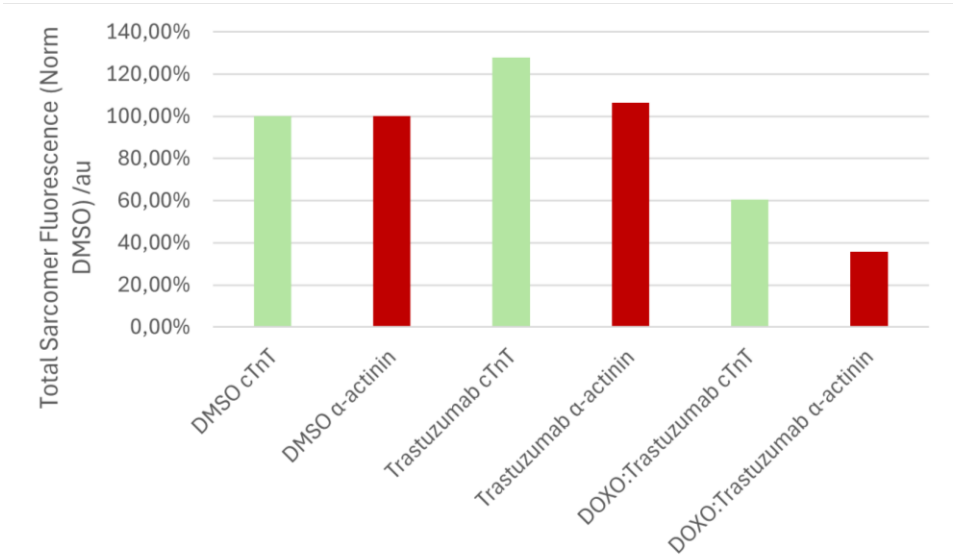


Figure 5. The total sarcomere fluorescence (TSF) of cTnT and *α*-actinin for the drug treatments 1 µM DMSO, 25 µM Trastuzumab and 1:1 µM DOXO:Trastuzumab normalized to the negative control DMSO, n=1.

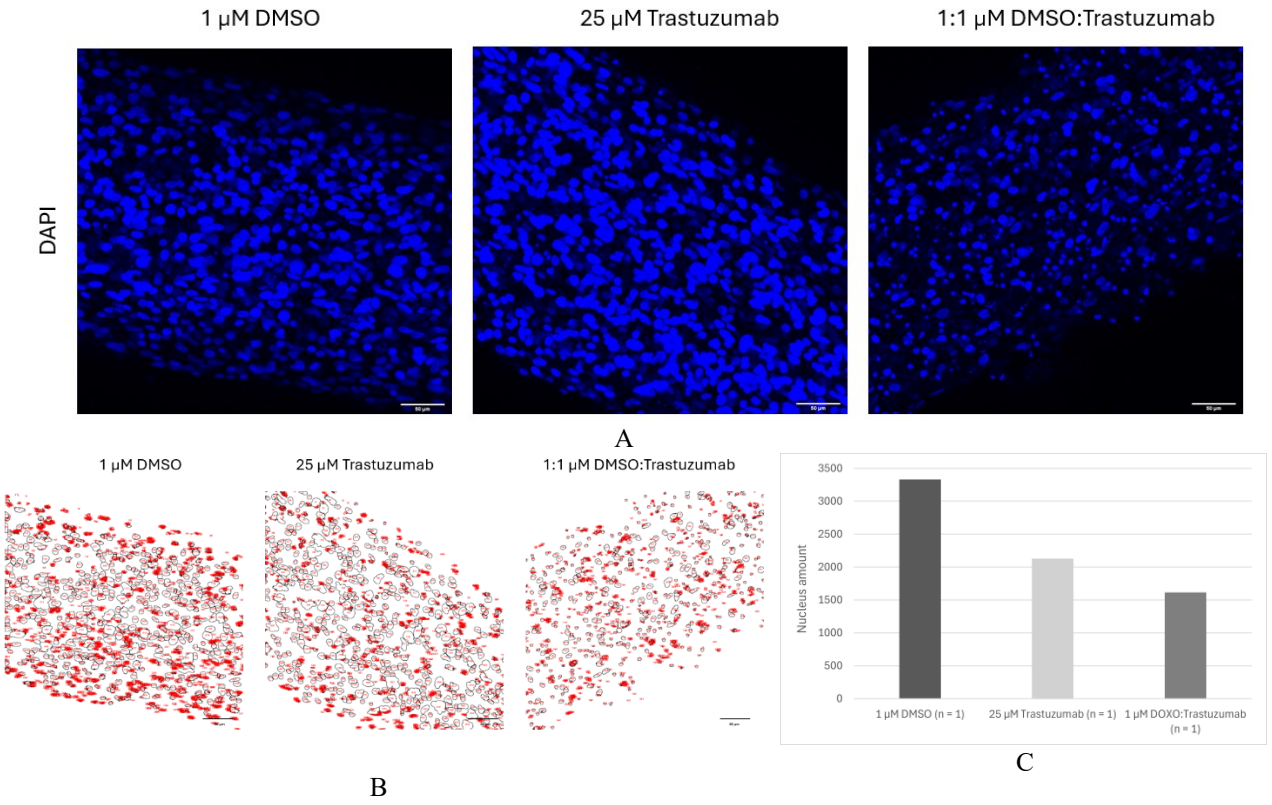


Figure 6. A) DAPI-staining for treated µ-engineered heart tissues with DMSO, 25 µM Trastuzumab and 1:1 µM DOXO:Trastuzumab. Scale bar = 50 µM.
B) Representative graphs of the nucleus counting in the DAPI staining for all the conditionswith the ImageJ software.
C) The amount of nuclei for the drug treatments 1 µM DMSO, 25 µM Trastuzumab and 1:1 µM DOXO:Trastuzumab with n = 1.

DISCUSSION AND CONCLUSION

Trastuzumab drug treatment in combination with anthracyclines has become a significant advancement in the treatment of HER2-positive breast cancer. However, the common side effect of cardiotoxicity has posed a major obstacle to this promising outcome. Developing an agent to protect against this severeside effect in the future would represent a substantial breakthrough in drug treatment for cancer. This requires experimental research with an advanced system to quantify these promising protective agents.Earlier studies have proved that 3D µEHTs are a rewarding platform for toxicity screening. This method supports the early recognition of cardiotoxic effects in compounds and effectively reproduces important facets of clinically relevant drug treatment protocols *in vitro*, considering peak dosing, repeated exposures, and recovery times. (4)
In this research, this has been confirmed with the cardiotoxic combination of DOXO and Trastuzumab,where after D7 a recovery in contractility takes place. Therefore, although the negative control DMSO isalso overreaching its original state and D10 is questionable, this study suggests that cardiac failure aftertreatment with the combination of DOXO with Trastuzumab has a recovery ability. However, the timeline of thisexperiment went up to D10, but it is still unclear if the tissues will keep recovering and if they willfully recover.

When the cardiomyocytes are constructing tissues after a few days they develop spontaneous beating. (17) To receive a more equal and constant frequency, we paced them at 1,5 Hz. (18) This was done to create the same conditions for every tissue. Unfortunately, at D10, the tissues did not follow the pacing. Although it is possible to electrically stimulate the EHTs, certain frequencies may not be captured when pacing rates are significantly lower or higher than the EHTs' spontaneous contraction rate. (19) It is feasible to pace them to beat faster, but notslower.

Similar to a previous study that has examined the toxicity for different concentrations of Trastuzumab compared to previous experience with DOXO, we observed that the concentration does not influence thecardiotoxicity of Trastuzumab. (7) Therefore, in this experiment Trastuzumab is not cardiotoxic *in vitro*. Nevertheless, we examined the FoC and the contraction kinetics. Which quantifies the disturbance in "cardiac rhythm", which is only one of the

identified major types of cardiotoxicity according to the research of Correspondence Pal Pacher MD. (20) Yet, there are three more major types of cardiotoxicity; “functional and structural heart impairment”, “arterial and venous thromboembolism” and “effects on blood pressure”. (20) Hence, this study has crossed one of the major types, “cardiac rhythm”, in clinicaltrials, one of the other major types might cause cardiotoxicity. In addition to this non-toxic outcome forTrastuzumab, we wondered if only DOXO is the cardiotoxicity factor. With this research, this hypothesis can not be excluded. Yet, previous clinical outcomes have already shown different proof, so broader *in vitro* research is acquired. (21)

For clinical treatment, it is also important to know how the cardiomyocytes are affected. For that reason, we have performed an immunostaining. The analysis like DAPI counting and the fluorescence intensity are based on only one image which makes it questionable if these observations are the case for the whole area. In the immunostaining images, the DMSO displayed sarcomeres as anticipated, indicating well-developed tissues. The Trastuzumab shows very similar results for the immunostaining which means that the critical factors for heart contraction, cTnT and *α*-actinin, are not affected. This is in line with the outcomes of the FoC and contraction kinetics. The lower intensity for the DOXO:Trastuzumab condition might demonstrate affection of the sarcomeres. Nevertheless, it is also possible that another factor in the cell was affected and this has led to a decrease of sarcomere formation. For this, we have a closer look into the α-actinin staining. The DOXO:Trastuzumab performance nuclei staining, whereas this effect is not observed with DMSO and Trastuzumab. This observation suggests that certain factors within the nuclei involved in sarcomere releasement may be affected as well. (22) Interestingly, for the DOXO with Trastuzumab condition, there is a difference in staining intensity between cTnT and *α*-actinin. They both fulfil distinct roles within sarcomeres. Cardiac troponin T (cTnT) and troponin I (cTnI) function as regulatory proteins within the heart, playing a crucial role in modulating the calcium-dependent interaction between actin and myosin. (23) cTnT, cTnI and cardiac troponin C interact closely and therefore affect the regulation and contraction role in cardiomyocytes. (24) On the contrary, α-actinin plays a pivotal role within the contractile apparatus, contributing significantly to mechanotransduction processes. (25) α-Actinin serves as a mechanosensitive protein, detecting mechanical stimuli and transducing them into biochemical signals that trigger specific cellular responses. (26). This is an interesting, but not strange outcome because the Erb2-signalling mechanism contains integrin-mediated mechanotransduction which is the cardiotoxic cause in Trastuzumab with anthracyclines. (27) Remarkable about this outcome is that this is not reflected in the Trastuzumab only condition. On the other hand, it also did not cause cardiotoxicity in the FoC and contraction kinetics.

Despite the significant and successful compounds this 3D platform consists of, there are still some difficulties with chip production. Despite, the large number (8 or 9) of chips at the start of the tissue seeding, both we ended up with only three chips with one or more beating tissues. In theory, the chip is designed that there is a gap of 10 µm between the top of the pillar and the glass. (17) Therefore, the tissues can form themselves nicely around the pillars and they can move the pillars while contracting. Sadly, during measurements, we discovered the DOXO with CPD chip has an attached pillar to the glass slide at the right side. (Figure 3A). This may explain the significant tissue loss observed before treatment. Due to the immobility of the pillar, the tissue might break or detach as a result of excessive tension. Another important aspect of the chip design for achieving the desired contraction is the pillar design with a length of 1 mm. (17) This length prevents the tissues from slipping of. However, during the production of the chips, bubbles may remain inthe PDMS. If these bubbles are located at the site of a pillar, they can affect its length and thus the tissuecan slip off. Adding to this, through the experience PDMS has resulted in a weak attachment to the glassslide and can cause leakage, several chips and tissues were lost. Given the limited number of samples inthe control groups, it was statistically unreliable to perform an overall normalization to the controls. Furthermore, the big loss of tissues has led to a very low amount of tissues and zero to very little replications. For a finer quantification, at least a triplicate is preferred which hopefully can be accomplished with microscopic research before adding the gell-cell suspension.

Nonetheless, the exceptional low generated 3D-µEHTs, we decided to perform a trial with the CPD. Due to a quantification of zero, it has only given a first indication of the effect of the CPD. Thus far, it seems that this concentration does not overrides the cardiotoxicity of the drugs yet. Where in the first experiment Trastuzumab with anthracycline was extensively cardiotoxic. In this experiment, the combined condition exhibits the lowest cardiotoxicity. Therefore, it would be intriguing for future research to investigate whether the CPD includes a protective mechanism against Erb2-signaling, which is the pathway implicated in cardiotoxicity induced by the combination of Trastuzumab with DOXO.

In conclusion, our findings confirm the cardiotoxicity of the combination therapy involving anthracyclines and Trastuzumab, with immunostaining revealing Erb2-signaling as the principal causative factor.Even, a high concentration of Trastuzumab alone did not significantly impact cardiotoxicity in our 3D-µEHT model. Furthermore, we created an indication that the CPD might not be cardioprotective in this experimental setting.

LIMITATIONS OF THE STUDY

One limitation of our study is that the results are based on only two hPSC lines which were available at this time of the research. In addition, they underwent drug treatment only at D1, while in clinical practice, patients often achieve drug treatments for severaldays. Another limitation is that we only analyzed one timepoint in our immunostaining instead of a timeline, which could be valuable for observing the sarcomere alteration from beginning to end. Further development of 3D heart models to include more of the identified major cardiac injuries with a broaderresearch platform would be of interest, especially for Trastuzumab. At last, further research on CPDs is necessary.

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