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BACHELOR THESIS

The effect of anthracyclines on the gene and protein expression of transcription factors IRX4 and HAND2 in cardiomyocytes

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Abstract

Anthracyclines, like Doxorubicin, are known to be cardiotoxic. This toxicity is for example caused by altered gene and protein expression which leads to apoptotic cell death. This research focuses on the effect of anthracyclines on the gene and protein expression of transcription factors IRX4 and HAND2. Aside from Doxorubicin, these effects are also research for new analogues of Doxorubicin; Amrubicin and Aclarubicin. In addition, the research also looks into the effect of cotreatment with Bortezomib on the gene and protein expressions of these transcription factors. This is evaluated by qPCR-based gene expression analysis and multiple immunostaining experiments for protein expression. These experiments showed that the transcription factor IRX4 is not affected by anthracyclines on both gene and protein level. The gene expression of HAND2 decreases after treatment with Doxorubicin and also the protein expression of HAND2 shows a decrease after treatment with Doxorubicin and Aclarubicin. The decrease of HAND2 proteins seems to increase with a higher concentration of anthracycline and with a longer treatment time. No conclusions can be drawn about the effects of Amrubicin treatment on HAND2 protein expression based on this research because of autofluorescence signal in the GFP channel caused by Amrubicin. The cotreatment with Bortezomib did not seem to have an effect on the gene and protein expression of IRX4 and does not prevent the downregulation of HAND2.

Contents

1 Introduction

Every year over a million cancer patients use anthracyclines such as Doxorubicin as chemotherapeutic agents. Doxorubicin effectively inhibits tumor cell proliferation in a variety of malignancies, however, these anti-cancer drugs can have severe side effects. They may cause therapy-related tumors but more important for this research, they may cause irreversible cardiotoxicity [1].

1.1 Damage mechanisms of Doxorubicin in cardiomyocytes

The underlying mechanisms of anthracycline-induced cardiotoxicity is not entirely understood but it is suggested that there are four major mechanisms of disease at play in the onset of cardiotoxicity: [2]

1. Mitochondrial dysfunction;
2. Disruption of calcium homeostasis;
3. DNA damage [3];
4. Altered gene and protein expression levels triggering apoptotic cell death.

Many different pathways are involved in anthracycline-induced cardiotoxicity and lead to the four factors mentioned above (see figure 1). Alteration of gene expression starts when Doxorubicin induces the release of HMGB1 (high-mobility group protein B1). This protein targets a membrane receptor (Toll-like receptor (TLR) 4) which then represses GATA4 (a transcription factor). This ultimately leads to inhibition of cardiac gene expression. Dependent on the function of the cardiac gene, inhibition can cause cell death [3].

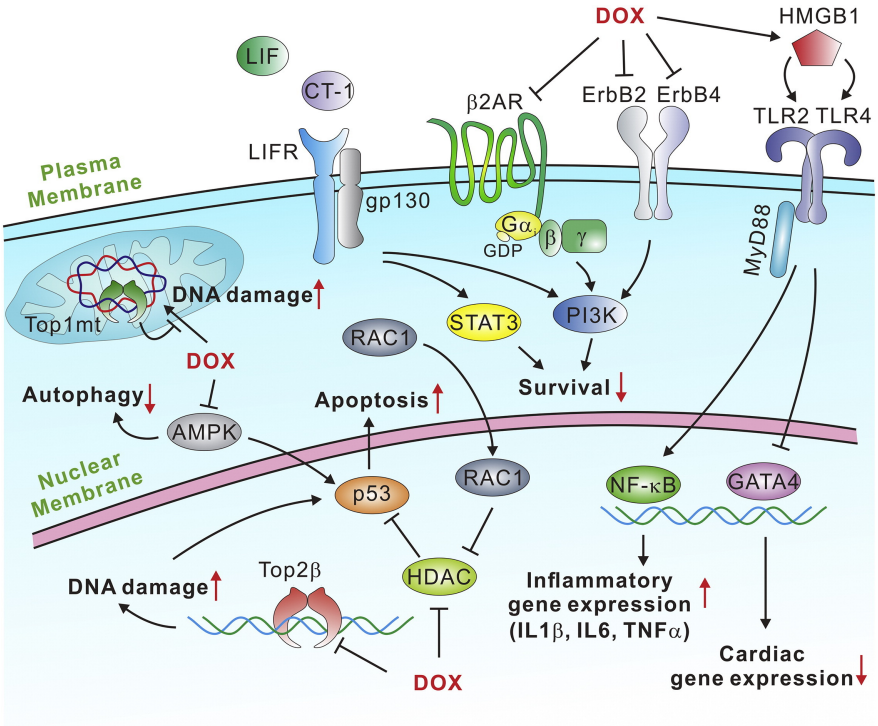


Figure 1: Overview of all the different pathways involved in anthracycline-induced cardiotoxicity.[3]

1.2 Analogues to Doxorubicin may alleviate cardiotoxicity

The toxicity of Doxorubicin has led to the development of chemical analogues, which may present a similar efficacy to combat malignant cells but prove less damaging to the heart. Two of these analogues are Amrubicin and Aclarubicin.

Amrubicin is a synthetic and quite recently developed anthracycline [4]. Just like with Doxorubicin, Amrubicin inhibits DNA topoisomerase II which leads to stabilizing topoisomerase II. This causes DNA double-strand breaks. However, Amrubicin in contrast to Doxorubicin shows almost no cardiotoxicity [4]. This difference in cardiotoxicity is most likely caused by a different pathway. Besides DNA damage, Doxorubicin also induces histone eviction. When this histone eviction is induced in promoter regions and gene body regions, this affects transcription of genes.

Aclarubicin, an analogue of Doxorubicin, also induces histone eviction but does not induce DNA double-strand breaks [5]. Even though, this anthracycline does not induce DNA double-strand breaks it shows similar cardiotoxicity as Doxorubicin.

1.3 Cardiac transcription factors IRX4 and HAND2

This research will focus on the gene and protein expression after treatment with anthracyclines. Earlier research within this research group has demonstrated a decrease of cardiac transcription factors NKX2.5 and MEF2C after Doxorubicin and Aclarubicin treatment. Based on these findings this research will look into two different transcription factor; IRX4 and HAND2.

IRX4 (Iroquois homeobox 4) is a plasma protein and transcription factor which can be found in cardiomyocytes in heart muscle tissue [6]. This protein is encoded by the IRX4 gene which can be found on chromosome 5. When encoded the protein can mainly be found in the nucleoplasm and in vesicles. The IRX4 protein is expected to be an important mediator of ventricular differentiation during heart development. IRX4 plays an important role in the heart by suppressing the atrial gene expression while possibly activating ventricular gene expression [7]. Because of this, loss of IRX4 in the heart disrupts ventricular properties which lead to cardiac decompensation.

HAND2 (Heart and neural crest derivatives expressed 2) is a transcription factor which is found in cardiomyocytes [8]. This protein is encoded by the HAND2 gene which belongs to the basic helix-loop-helix family of transcription factors and can be found on chromosome 4. When encoded the protein is mainly found in the nucleoplasm. The HAND2 protein plays an important role in the development of the heart, especially in the development of the right ventricle and the aortic arch arteries. This protein is also essential for the formation and regulation of angiogenesis. After cardiomyocytes are exposed to Doxorubicin a rapid depletion of the HAND2 transcription factor can be seen in cultured cardiomyocytes [9]. In mice cardiomyocytes depletion of HAND2 causes hypoplasia which is the result of excessive programmed cell death [10].

1.4 Bortezomib might prevent downregulation of transcription factors

This research also looks at the effects of Bortezomib on the gene and protein expression of HAND2 and IRX4 after treatment with anthracyclines.

Bortezomib is an anti-cancer drug. It is a proteasome inhibitor which means it inhibits the protein complexes that break down unneeded or damaged proteins [11][12]. Proteosomes are present in cancer cells but also in normal cells. However, the level of proteasome activity in cancer cells is higher than in normal cells which makes cancer cells more sensitive to proteasome inhibition. Bortezomib is useful as anti-cancer drug because targeting the proteosomes leads to a build-up of unwanted proteins. This eventually causes the cells to die.

It is suggested that cotreatment with Bortezomib can prevent a decrease of cardiac transcription factors. Bortezomib specifically inhibits the protease activity of the 26S proteasome [13]. Poizat et al. has shown that treatment with Doxorubicin promotes breakdown of the p300 transcription factor in rat cardiomyocytes by the 26S proteasome. P300 is a transcription factor and coactivator of MEF2C, which is upstream of HAND2 [9]. This suggests that by adding cotreatment with Bortezomib this breakdown of p300 can be prevented, which ultimately leads to prevention of HAND2 knock-down.

This research focuses on the altered gene and protein expression of cardiac transcription factors IRX4 and HAND2 caused by anthracyclines. Therefore the first research question is:
How do anthracyclines affect the gene and protein expression of the transcription factors IRX4 and HAND2 in cardiomyocytes?

The second question for this research is based on the suggestion that Bortezomib can prevent expected knock-down of HAND2. So the second research question is:
How does Bortezomib affect the gene and protein expression of the transcription factors IRX4 and HAND2 in cardiomyocytes after treatment with anthracyclines?

2 Materials and methods

2.1 Cell maintenance and drug exposure

Doxorubicin was obtained from Sigma-Aldrich (D1515). Aclarubicin (sc-200160) and Amrubicin (sc-207289) were a kind gift from the Neefjes lab at Leiden University Medical Center, which obtained these drugs from Santa Cruz Biotechnology. Bortezomib (5.04314) was purchased from Sigma-Aldrich. These drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650), Doxorubicin at 10 M, Amrubicin and Aclarubicin both at a concentration of 1 M and Bortezomib at 1 mM. Subsequently, these drugs were aliquoted and stored at -20°C for further use. The final concentrations of the anthracyclines varied between 1 and 10 μM and of Bortezomib between 0.1 and 1 μM. DMSO was used as control condition at a concentration of 10 μM.

hiPSC (LUMC0020iCTRL-06) were differentiated towards ventricular cardiomyocytes [14], after which the cardiomyocytes were cryo-preserved until further use. For this study, iPSC-cardiomyocytes were seeded as monolayers at a density of 90 K cells per cm^2 into CellCarrier-96-well special optics plates (PerkinElmer) and at 130 K cells per cm^2 into a 12-well plate for RT-qPCR, coated with fibronectin (5 μg/ml, F1141, Sigma-Aldrich) and Matrigel (65 μg/ml, 65354230, Corning). For cell culture, cardiomyocyte medium [14] with 0.25 MW% bovine serum albumin (BSA) (A9418, Sigma-Aldrich) was supplemented with triiodothyronine hormone (100 nM, Sigma-Aldrich), synthetic glucocorticoid dexamethasone (1 μM, Sigma-Aldrich) and insulin-like growth factor 1 (100 ng/ml, Long R3 IGF-1, Sigma-Aldrich) (TDI) for improved maturity of cardiomyocytes [14]. The medium was refreshed every 3-4 days. Cells were allowed to recover for at least 10 days after cell seeding before drug treatment was initiated. For the immunohistochemistry assessment, the cells were treated with varying concentrations of anthracyclines for varying time periods.

- Timelapse: the cells were treated with 1 μM, 5 μM and 10 μM of Doxorubicin for 0, 2, 4, 6, 8 hours and with 1 μM, 5 μM and 10 μM of Doxorubicin and 5 μM and 10 μM of Amrubicin and Aclarubicin for 24 hours.
- 3 week wash out: the cells were treated with 1 μM, 5 μM and 10 μM of Doxorubicin for 24 hours, after which the medium was refreshed, and the cell culture continued in cardiomyocyte medium supplemented with TDI.
- Bortezomib cotreatment: the cells were first treated with 0.1 μM and 1 μM of Bortezomib for 30 minutes. This is followed by 24 hours of treatment with 1 μM, 5 μM and 10 μM of Doxorubicin and 5 μM of Amrubicin and Aclarubicin.

During cell culture, the cardiomyocytes were maintained in a humified atmosphere of 5% CO2 at 37 °C that was regularly tested for the absence of mycoplasma.

2.2 RNA isolation, cDNA synthesis and quantitative PCR

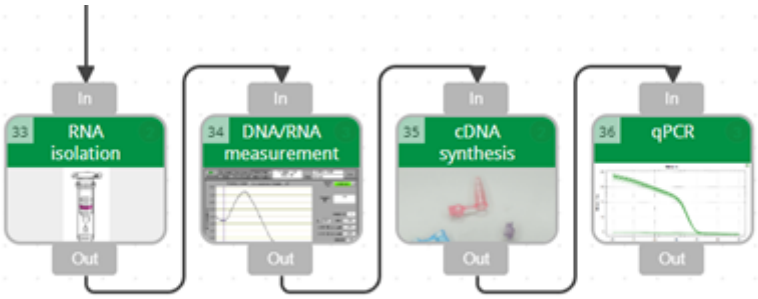


Figure 2: Method used for qPCR.

After treatment, cells were lysed and mRNA was isolated with the NucleoSpin RNA kit (740955.50, Macherey-Nagel). The mRNA concentration was measured with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The iScript cDNA synthesis kit (1708891, Bio-Rad) was used for cDNA synthesis, and this was performed in the T100 Thermal Cycler (Bio-Rad). Lysed cell samples and extracted RNA samples were stored at -80 °C. For the reverse transcription PCR (RT-qPCR) procedure, the SensiMix SYBR & Fluorescein Kit (QT615-05, Meridian Bioscience) was used, and this reaction was performed in the C1000 Touch Thermal Cycler (Bio-Rad). Primers used were: HAND2 (fwd: ACATCGCCTACCTCATGGAC, rev: TGGTTTTCTTGTCGTTGCTG) and IRX4 (fwd: ATGCTTCAGGGTATCTGGCCTCTT, rev: TTGGACTCCTGGGAACATGGACAA). hARP (fwd: CACCATTGAAATCCTGAGTGATGT, rev: TGACCAGCCCAAAGGAGAAG) was used as a housekeeping gene and fold changes in gene expression were calculated relative to the DMSO condition. Results obtained from the qPCR were processed in BioRadCFXManager software.

2.3 Immunohistochemistry

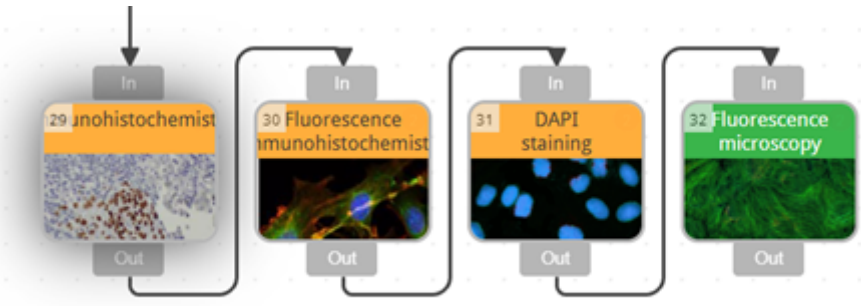


Figure 3: Method for immunohistochemistry

After treatment, cells were fixated with formaldehyde 4% (252549, Sigma-Aldrich) and permeabilized with 0.1% TritonX100 (Sigma-Aldrich, T8787), followed by blocking non-specific binding with 1 MW% BSA and 10% normal goat serum (Sigma-Aldrich, G9023) diluted in phosphate buffered saline (PBS) (17-516F, Lonza). Antibodies were diluted in PBS supplemented with 1 MW% BSA and incubated overnight. Primary antibodies used were IRX4 (1:200, rabbit pAb 104135-T34, Sino Biological), dHAND (1:200, mouse mAb sc-398167, Santa Cruz). After incubation with the primary antibodies, cells were washed 3x with PBS followed by 60-120 minutes incubation with the secondary antibodies. Cy5 anti-rabbit (1:400, goat IgG A10523, Thermo Fisher Scientific) and GFP

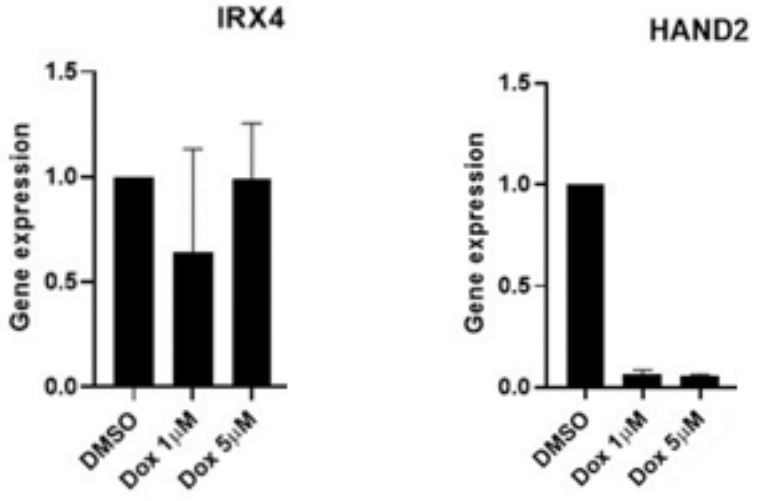
anti-mouse (1:400, goat IgG A11001, Thermo Fisher Scientific) were used as secondary antibodies. Lastly, DAPI (1:10000, D1306, Thermo Fisher Scientific) was added. The plates were kept at 4°C in PBS until further use. ±6 pictures were taken per well.

The EVOS FL Auto 2 (Thermo Fisher Scientific) equipped with a mercury light was used to image the cells treated with immunohistochemistry with Cy5 (exc: 628/40nm, emi: 685/40nm), GFP (exc: 482/25nm, emi: 524/24nm) and DAPI (exc: 357/44nm, emi: 447/60nm) filter cubes at 40x magnification. Doxorubicin has intrinsic fluorescent properties and was visualized with the RFP (exc: 531/40nm, emi: 593/40nm) cube. The images were further processed with ImageJ software. The quantification of fluorescent signal was performed by a custom ImageJ macro script.

3 Results

3.1 HAND2 gene, but not IRX4, is downregulated upon exposure to Doxorubicin

IPSC-cardiomyocytes were exposed to 1 μM and 5 μM of Doxorubicin for 24 hours. After this the cells were lysed and after RNA isolation and cDNA synthesis a qPCR was done (figure 4). Figure 4a shows no change in gene expression of IRX4 after treatment with Doxorubicin. Figure 4b shows that the cells treated with Doxorubicin show a substantial decrease in HAND2 gene expression. Also, it shows no difference in treatment with 1 μM or 5 μM of Doxorubicin.



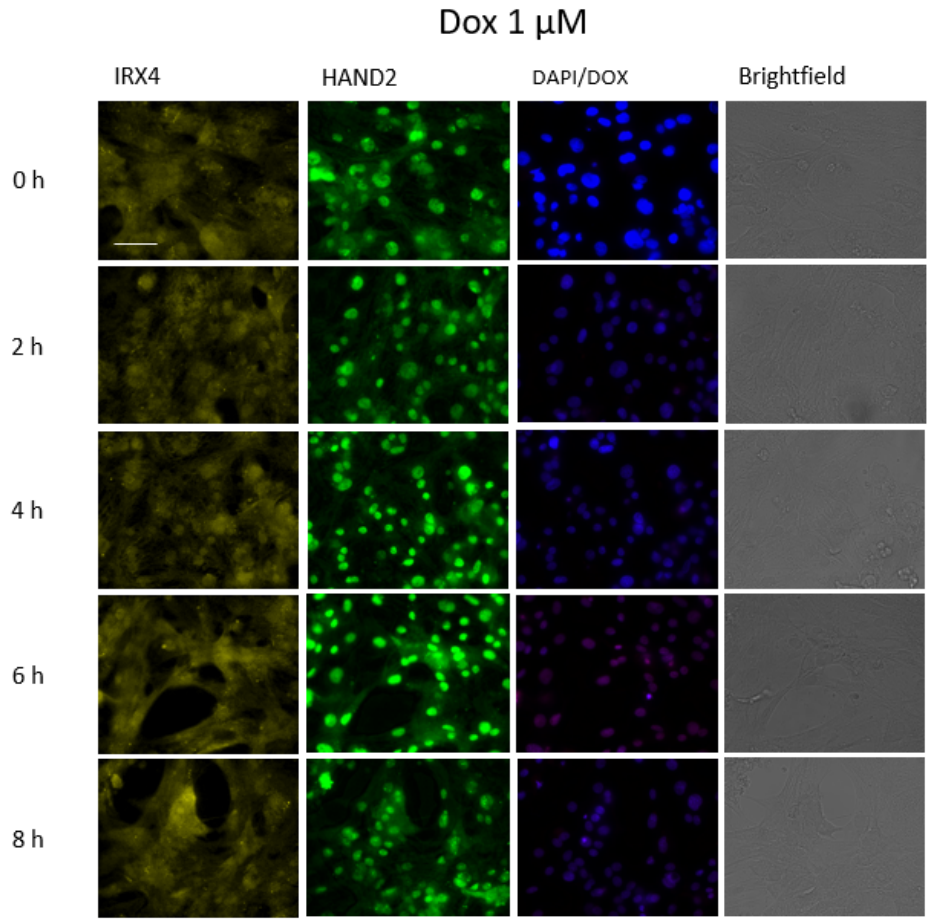
(a) *(b)*

Figure 4: The graphs show the gene expression of IRX4 (a) and HAND2 (b) after treatment with Doxorubicin. The cells were treated with 0 μM (DMSO), 1 μM and 5 μM of Doxorubicin.

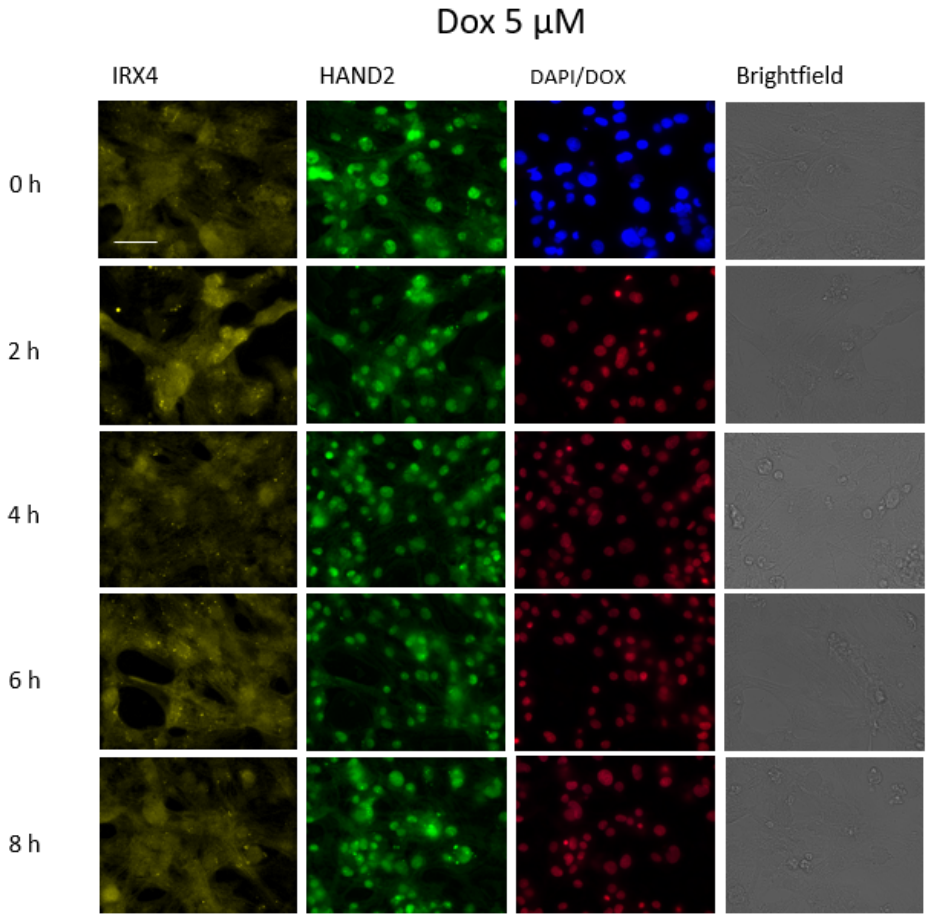
3.2 Protein expression of HAND2 decreases in first 8 hours of Doxorubicin treatment

We sought to investigate the kinetics of transcription factor depletion found in our qPCR results, by exposing iPSC-cardiomyocytes to several Doxorubicin concentrations and fix the cells in an hourly interval. The timelapse experiment consists of two parts. The first part looks at the effect of 1 μM, 5 μM and 10 μM of Doxorubicin after treatment for 0, 2, 4, 6 and 8 hours. The second part looks at the effect of 1 μM, 5 μM and 10 μM of Doxorubicin and 5 μM and 10 μM of Amrubicin and Aclarubicin after treatment for 24 hours.

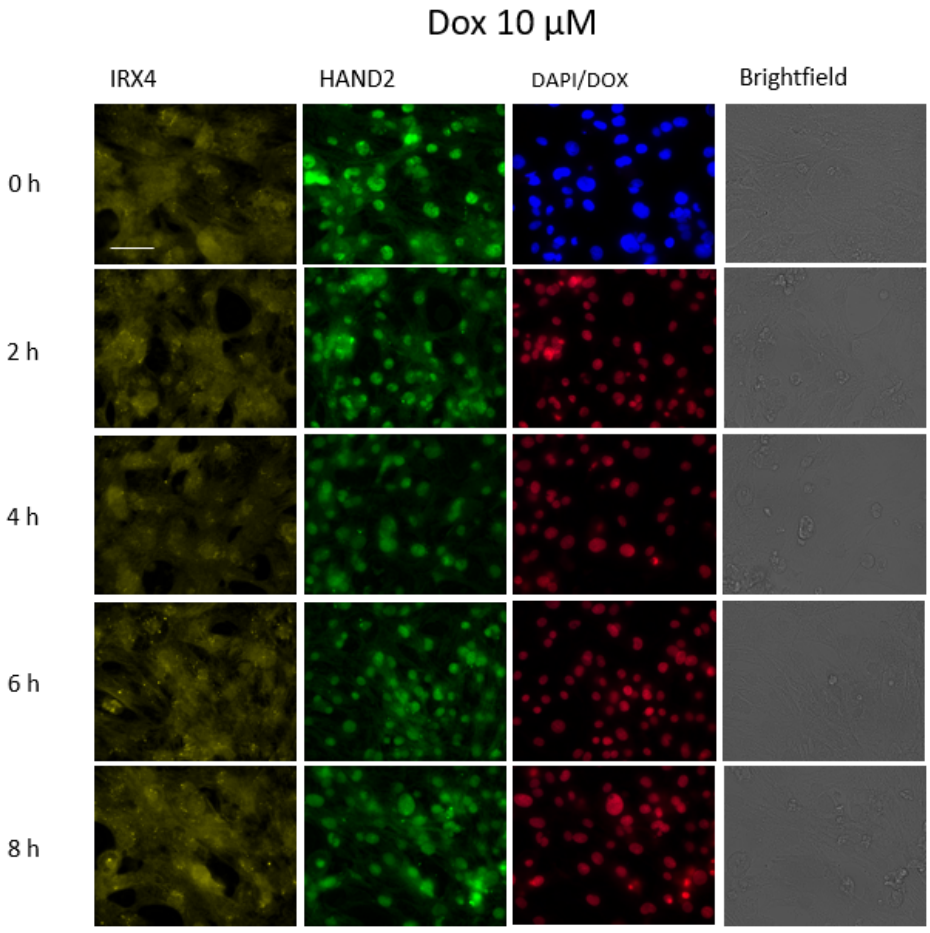
Figure 5a shows that both the cytosolic IRX4 and the nuclear HAND2 signal do not seem to decrease over time when 1 μM of Doxorubicin is added to the cells. After treatment with 5 μM and 10 μM of Doxorubicin, the fluorescent intensity of the IRX4 label remains comparable with each other and with the IRX4 signal after treatment with 1 μM. However, the HAND2 signals demonstrate a reduction in fluorescence over time. Unexpectedly, the HAND2 signal after treatment with Doxorubicin seems to have a similar intensity for treatment with different concentrations.
Fluorescent intensity was further quantified (figure 5d) by custom software analysis. The quantification confirms a downward trend of fluorescent intensity at Doxorubicin concentrations of 5 μM and 10 μM over time, and a reduction of intensity after 8 hours for the Doxorubicin 1 μM group. However, it also shows that the intensity of the HAND2 signals remain visible up to 8 hours after Doxorubicin exposure, indicating that HAND2 remains present in the nucleus.


Dox 1 µM
IRX4
HAND2
DAPI/DOX
Brightfield
0 h
2 h
4 h
6 h
8 h

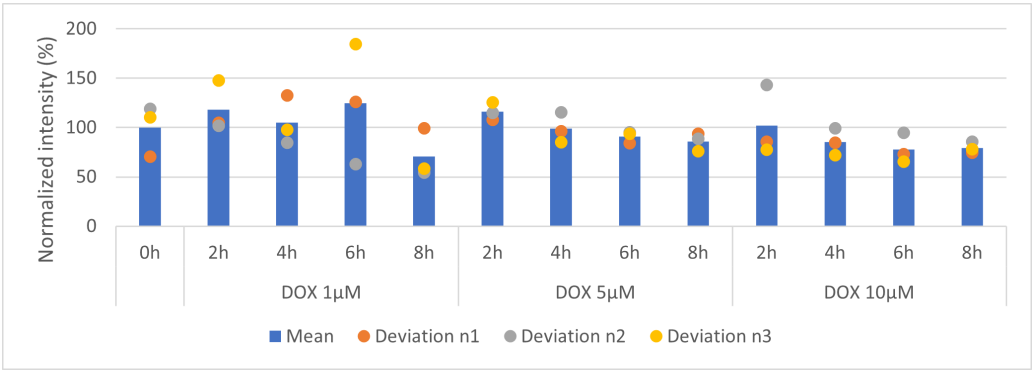
(a)


Dox 5 µM
IRX4
HAND2
DAPI/DOX
Brightfield
0 h
2 h
4 h
6 h
8 h

(b)



(c)



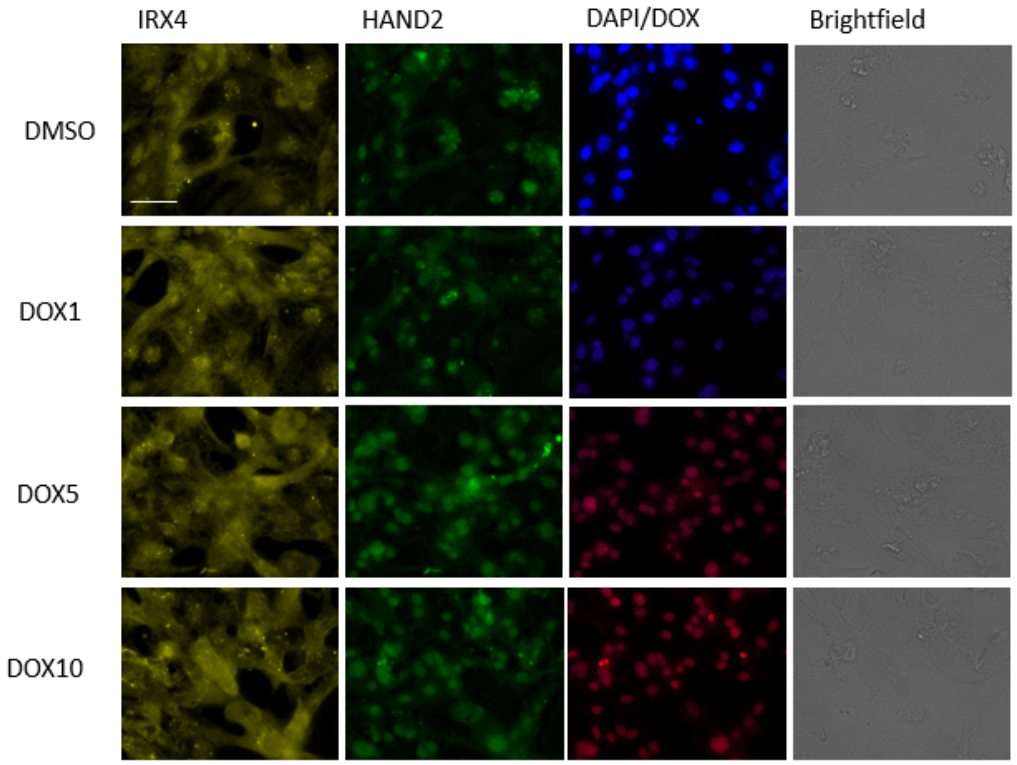
(d)

Figure 5: The top figures show microscopic images of different signals; the IRX4, HAND2, DAPI/DOX and brightfield signals. These images are made after treatment for 0, 2, 4, 6 or 8h with different Doxorubicin concentrations; 1 µM (a), 5 µM (b) and 10 µM (c). The scale bar shown is the same for every picture and has a length of 100 µm. The quantification of the HAND2 results is shown in (d).

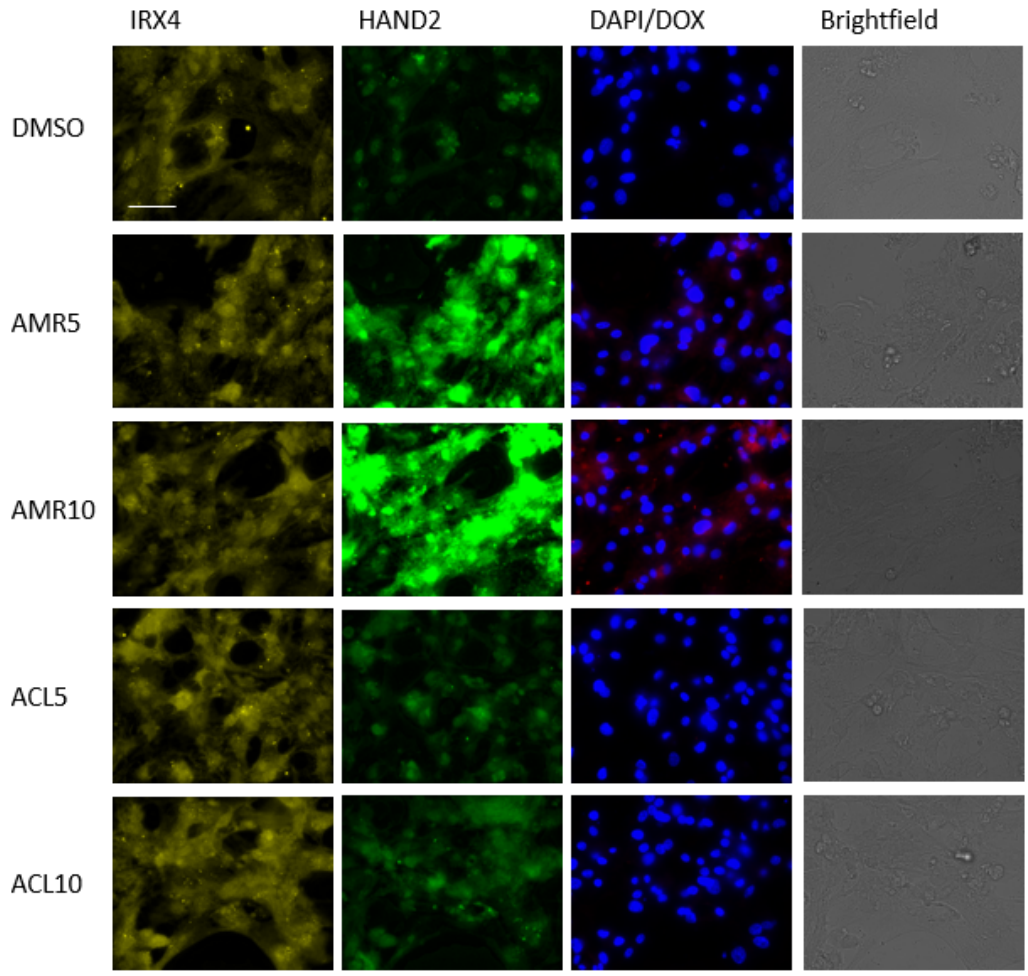
The 24 hour experiment (figures 6a and 6b) shows a very low intensity of the HAND2 signal in the untreated cells (DMSO condition). Unexpectedly, the HAND2 signal in figure 6a seems to increase after treatment with an increasing concentration of Doxorubicin. The IRX4 signal does not seem to change after treatment with Doxorubicin. Figure 6b shows cells treated with Amrubicin and Aclarubicin. Here it can be seen that treatment with both concentrations of Amrubicin cause a very strong signal in the GFP channel. This signal can be seen in the entire cells instead of in the nuclei as expected. The cells treated with Aclarubicin show a much lower signal than the Amrubicin-treated

cells but a similar signal as the untreated cells. Also after treatment for 24h with these anthracyclines the IRX4 intensity does not seem to change.

Quantification of these results shows the same results as seen in the images (figures 6a and 6b). Here it can be seen that indeed the intensity is very high in the Amrubicin-treated cells compared to all the other conditions and that the intensity of HAND2 increases with an increasing concentration of Doxorubicin. Also, in this graph the Aclarubicin-treated cells show a similar intensity as the untreated cells.



(a)



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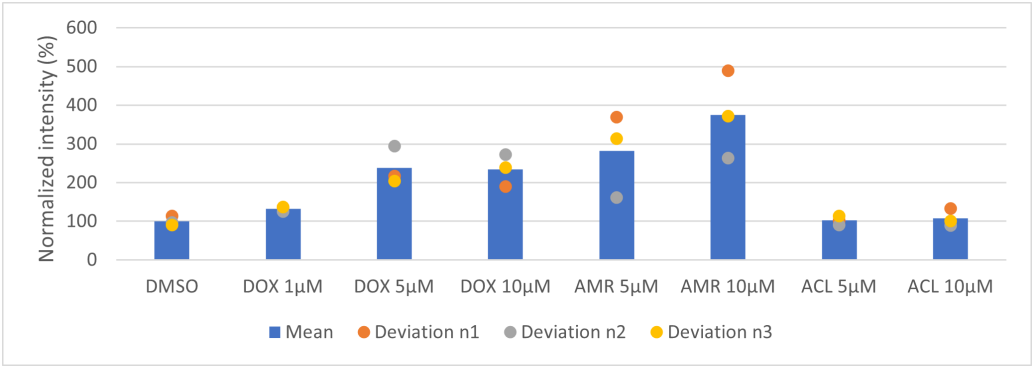


(c)

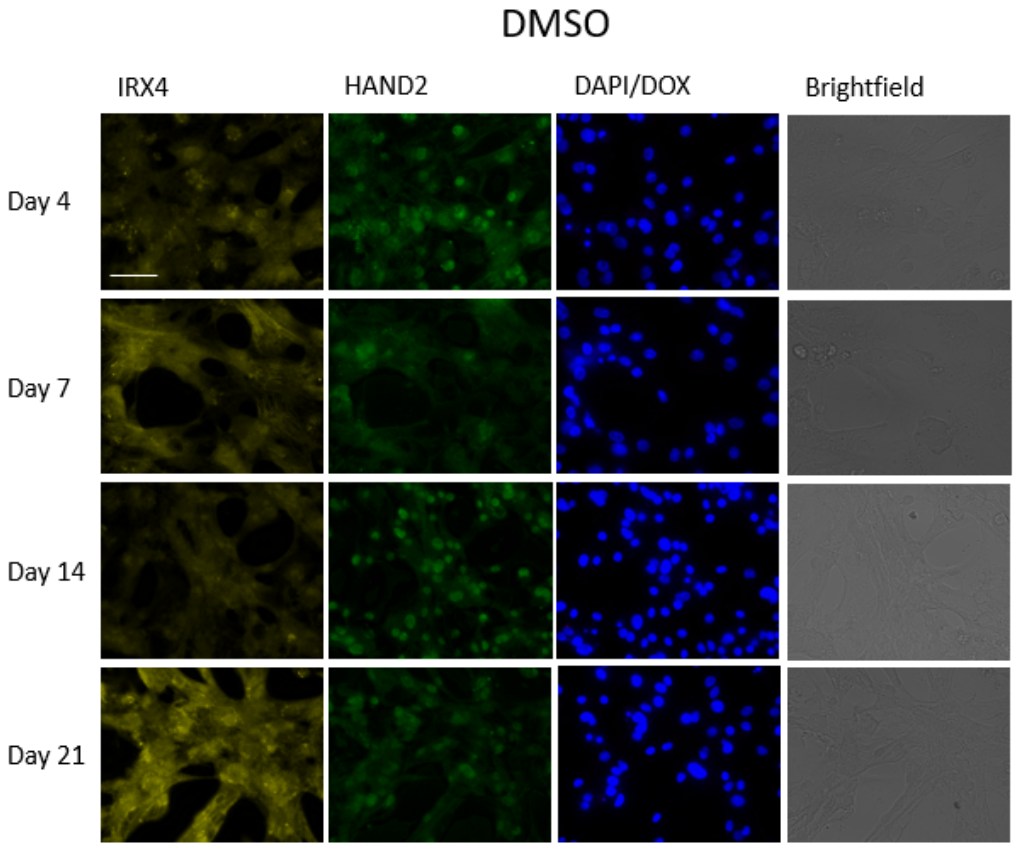
Figure 6: The top figures show microscopic images of different signals; the IRX4, HAND2, DAPI/DOX and brightfield signals. These images are made after treatment for 24h with different anthracyclines and different concentrations; 1 µM, 5 µM and 10 µM of Doxorubicin (a), 5 µM and 10 µM of Amrubicin and 5 µM and 10 µM of Aclarubicin (b). The scale bar shown is the same for every picture and has a length of 100 µm. The quantification of the HAND2 results is shown in (c).

3.3 Cardiomyocytes do not survive a long timelapse with high concentrations of Doxorubicin

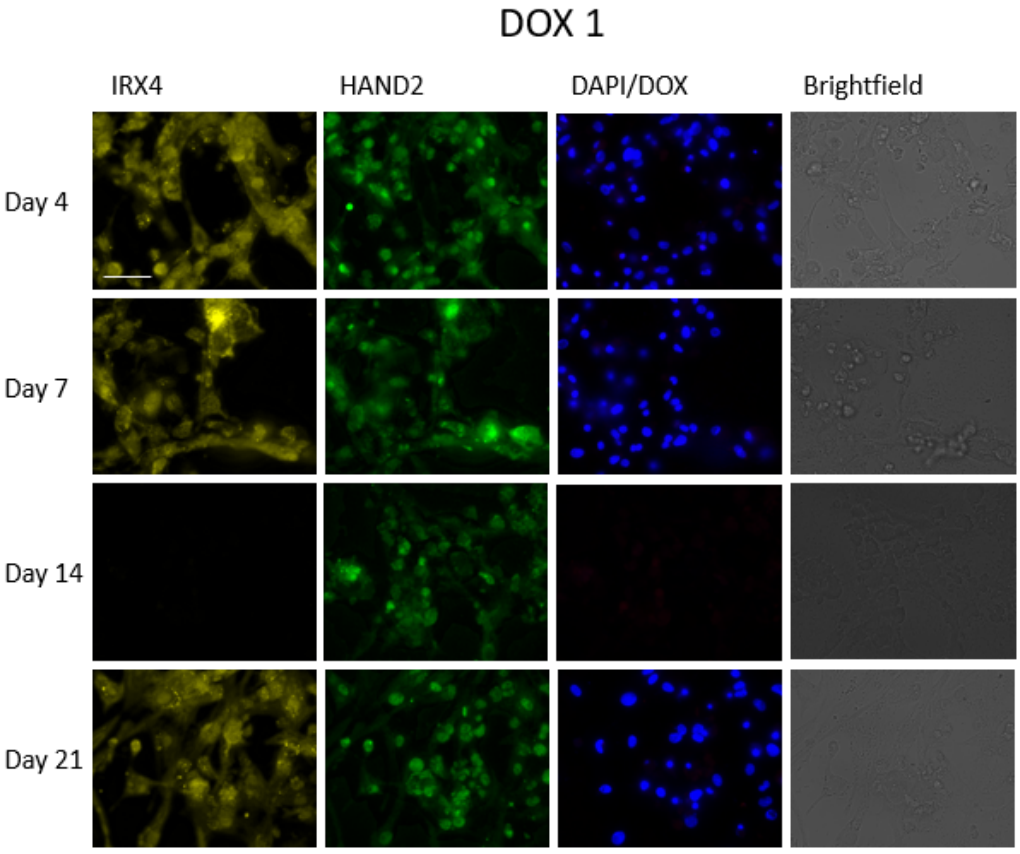
To determine the duration of the knock-down of HAND2 following Doxorubicin exposure to the iPSC-cardiomyocytes, we treated the cells with Doxorubicin for 24h. After this they were allowed to recover for a maximum of 21 days.After 4, 7, 14 and 21 days the cells were imaged (figure 7).

A very low intensity of IRX4 and HAND2 could be observed in the untreated cells (figure 7a). This does not seem to change over time. The cells treated with 1 μM, 5 μM and 10 μM of Doxorubicin seem to show more signal for both proteins (figures 7b to 7d). Unexpectedly, after treatment with high concentrations of Doxorubicin, the intensity of IRX4 seems to decrease over time while the HAND2 images show very strong signals after treatment with 5 μM and 10 μM. The brightfield pictures of these conditions indicate that these cells are no longer viable. It also stands out that there are no results 14 days after treatment with 1 μM of Doxorubicin. The IRX4 and DAPI/DOX images show no signal. The HAND2 image shows a weak signal.

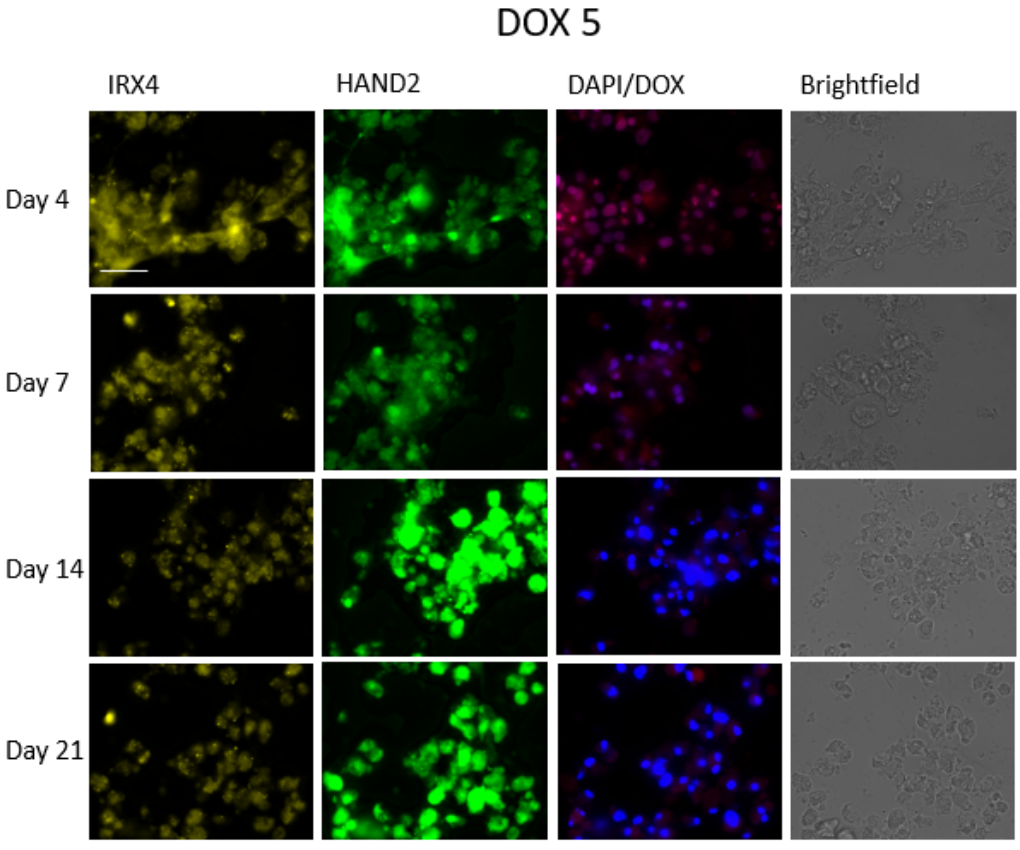
After quantification (figure 7e), the data shows again a much higher intensity for HAND2 in cells treated with 5 μM and 10 μM than in untreated cells or cells treated with 1 μM. It also shows little difference in the intensity in untreated cells or cells treated with 1 μM and over time. Furthermore, here it also shows that there are no results 14 days after treatment with 1 μM of Doxorubicin.



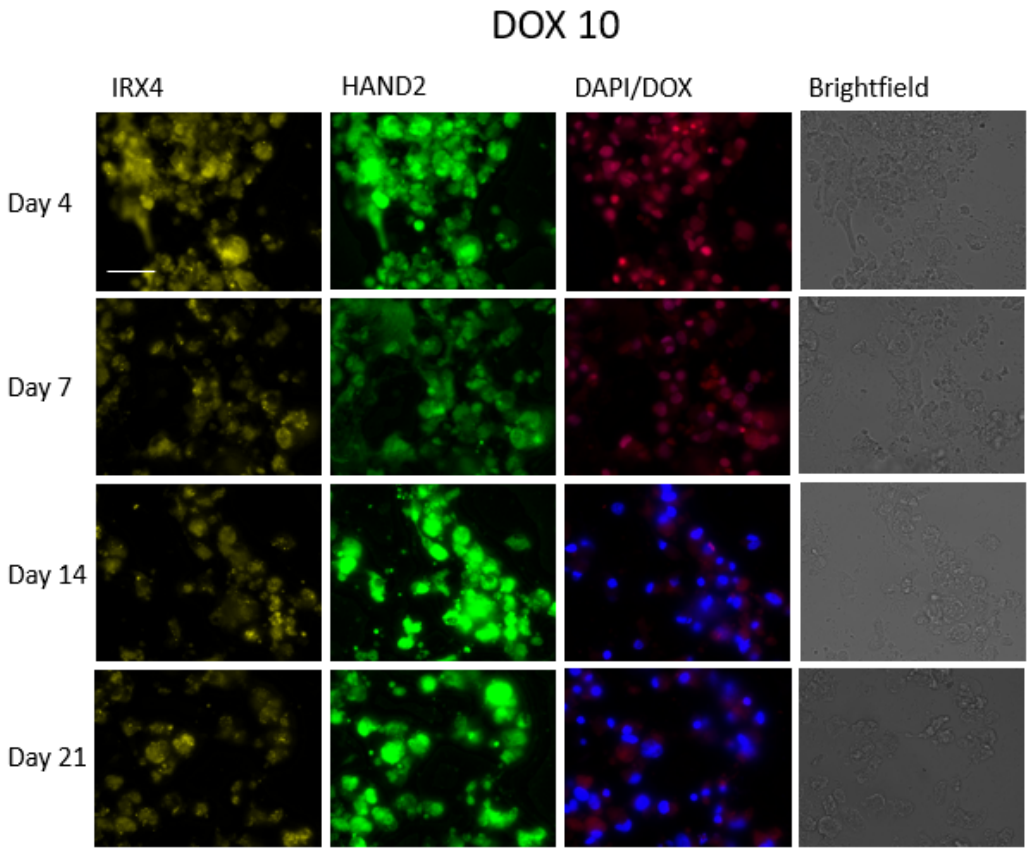
(a)


DOX 1
IRX4
HAND2
DAPI/DOX
Brightfield
Day 4
Day 7
Day 14
Day 21

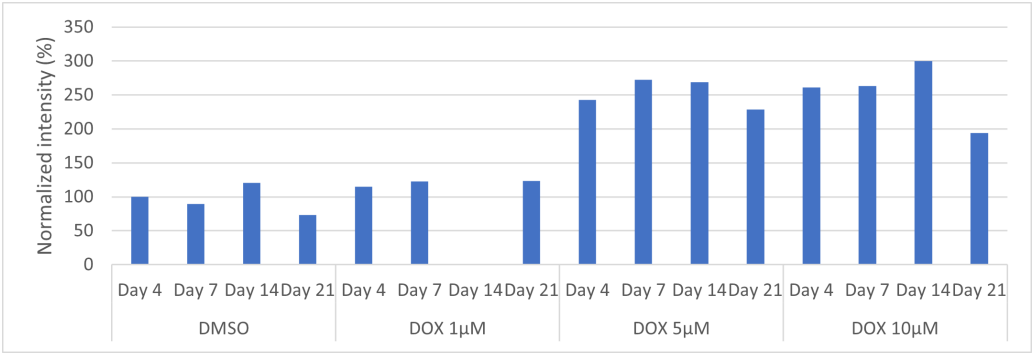
(b)


DOX 5
IRX4
HAND2
DAPI/DOX
Brightfield
Day 4
Day 7
Day 14
Day 21

(c)



(d)



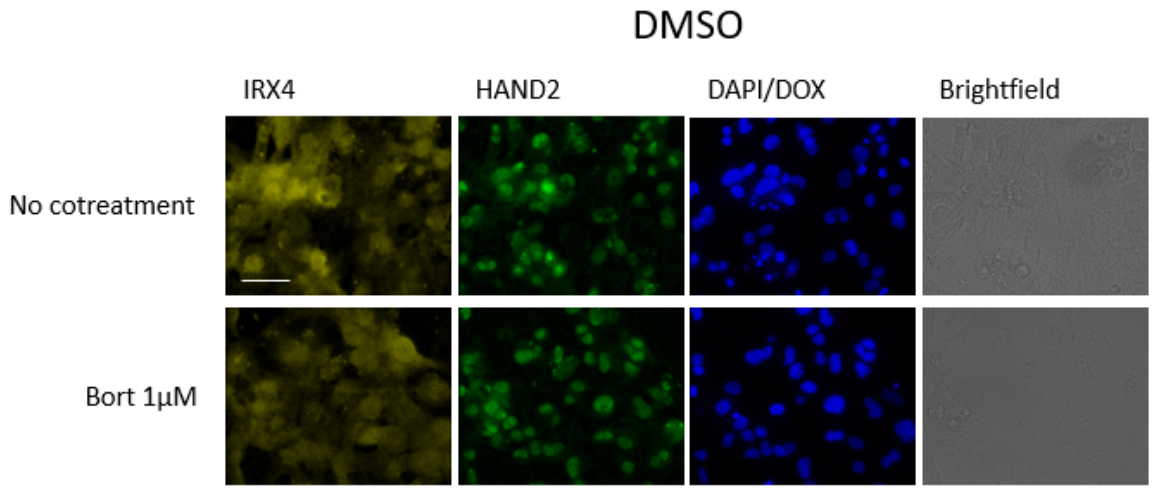
(e)

Figure 7: The top figures show microscopic images of different signals; the IRX4, HAND2, DAPI/DOX and brightfield signals. These images are made 4, 7, 14 and 21 days after 24h of treatment with different concentrations of Doxorubicin; 0 µM (DMSO) (a), 1 µM (b), 5 µM (c) and 10 µM (e). The scale bar is the same for every picture and has a length of 100 µm. The quantification of the HAND2 results is shown in (e).

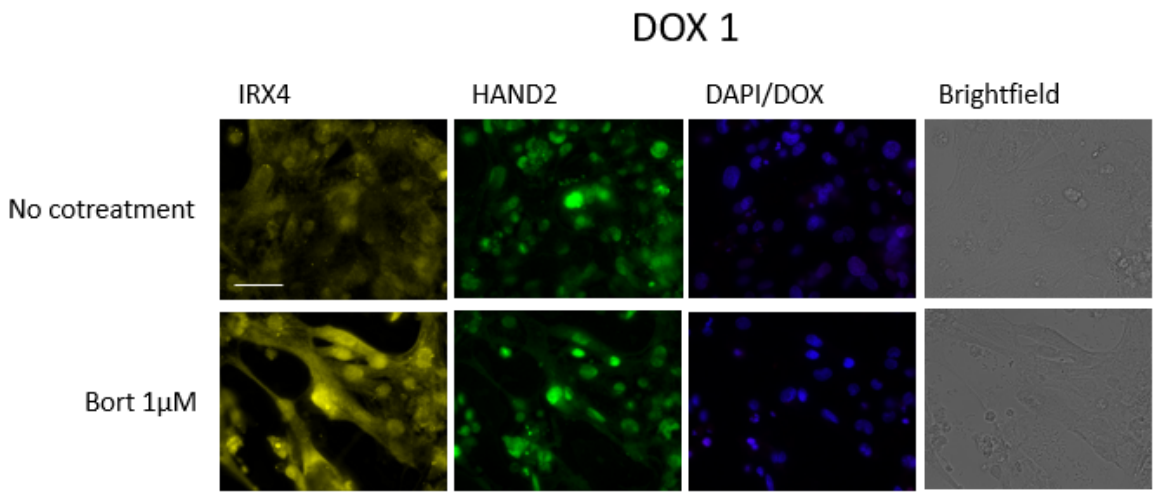
3.4 Bortezomib does not prevent downregulation of HAND2

To see if the downregulation of HAND2 by Doxorubicin exposure can be prevented, a cotreatment of 1 μM of Bortezomib is added for half an hour before treatment with anthracyclines for 24 hours.

Figure 8 does not show a big change in intensity of IRX4 or HAND2 when cotreatment of Bortezomib is added. Compared to the untreated cells (figure 8a) the HAND2 and IRX4 signal look similar after treatment with Aclarubicin (figure 8f) and stronger when the cells are treated with Doxorubicin (figures 8b to 8d). The cells treated with Amrubicin (figure 8e) again show a much stronger signal in the HAND2 images than the untreated cells. The IRX4 signal after treatment with Amrubicin seems to be similar to the IRX4 signal in untreated cells.
The graph in figure 8g shows similar results for HAND2 as can be seen in figures 8a to 8f.



(a)



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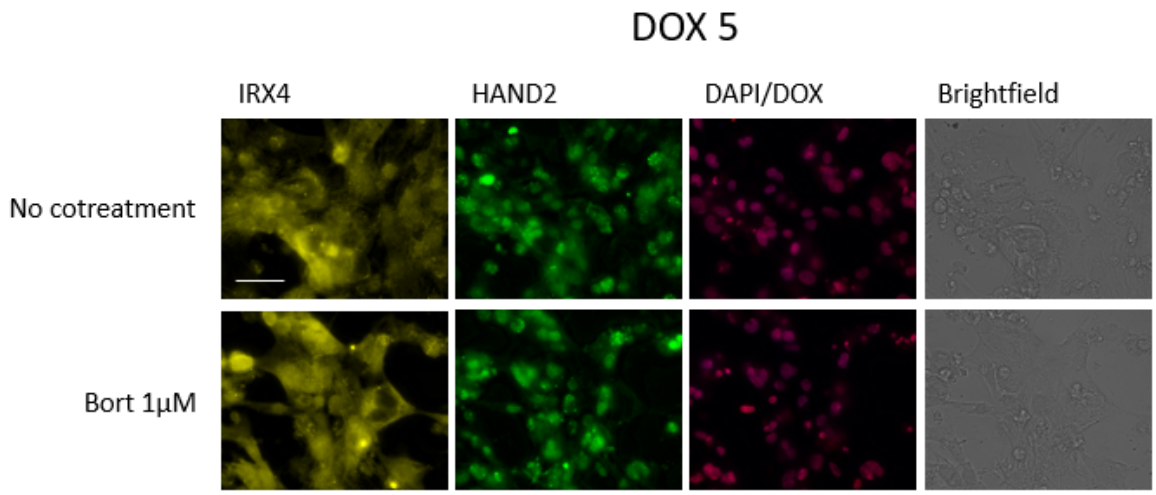
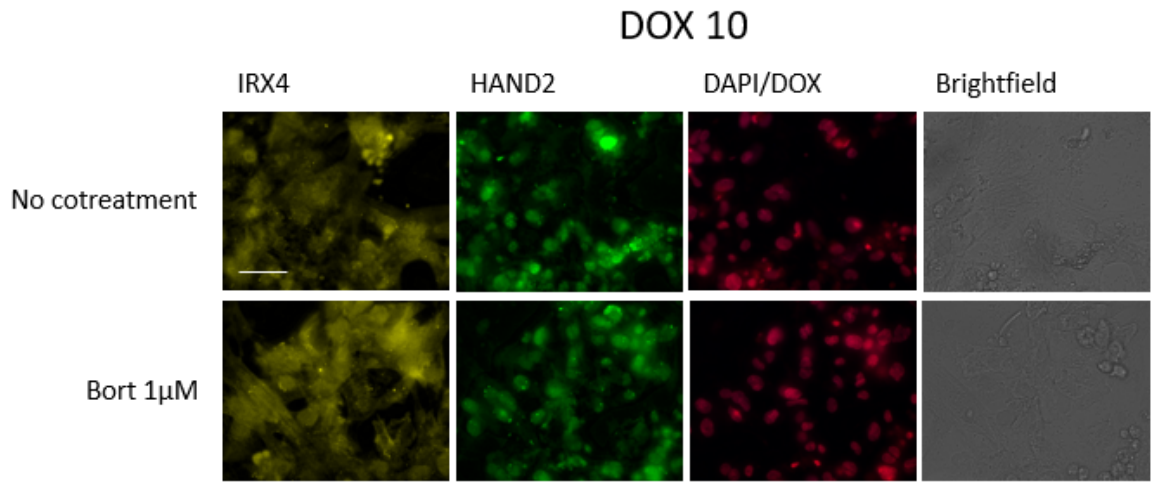
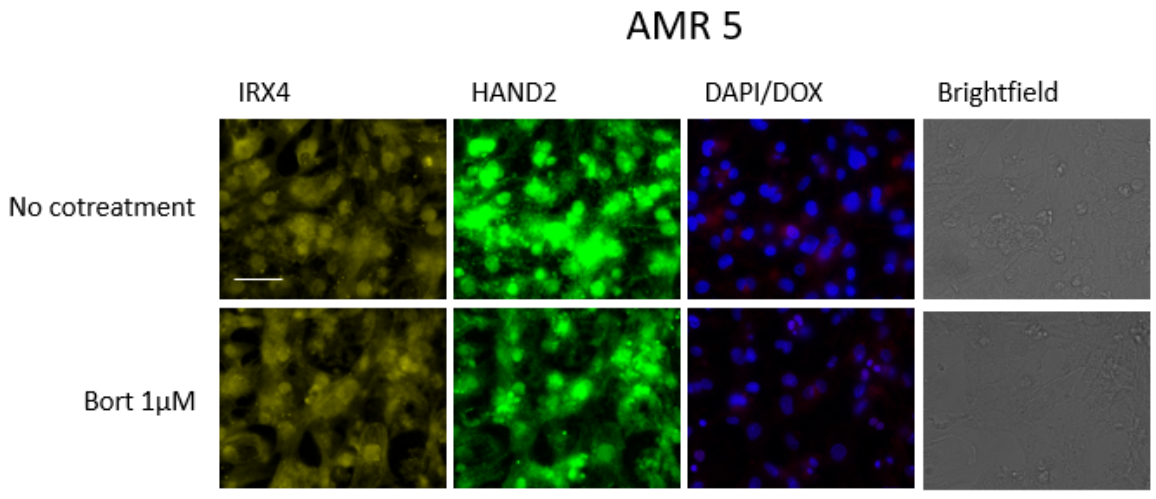

DOX 5
IRX4
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 1µM

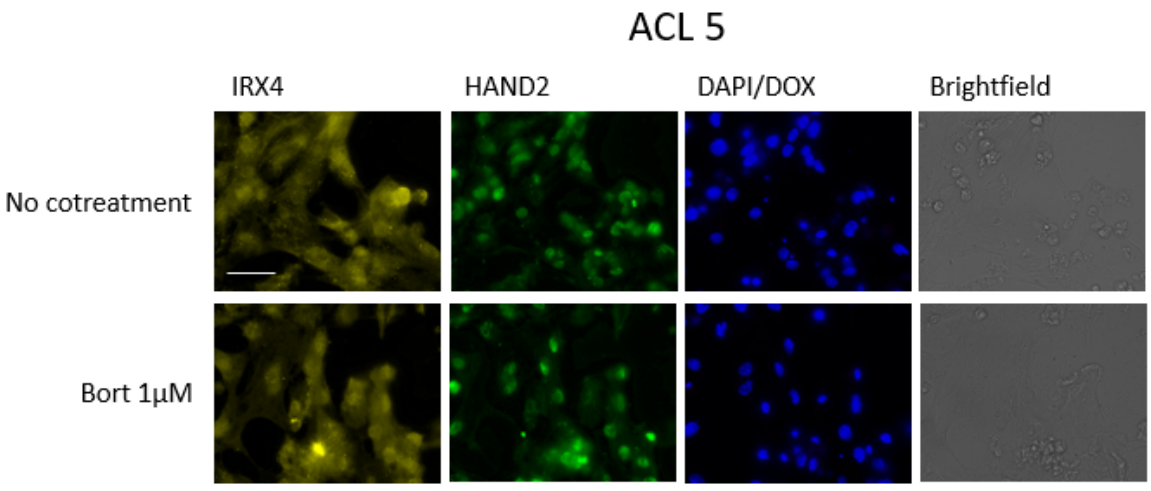
(c)


DOX 10
IRX4
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 1µM

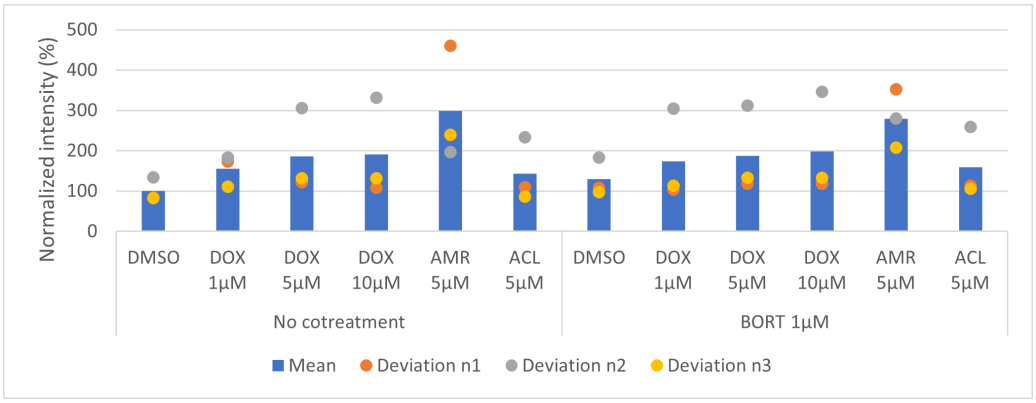
(d)


AMR 5
IRX4
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 1µM

(e)



(f)



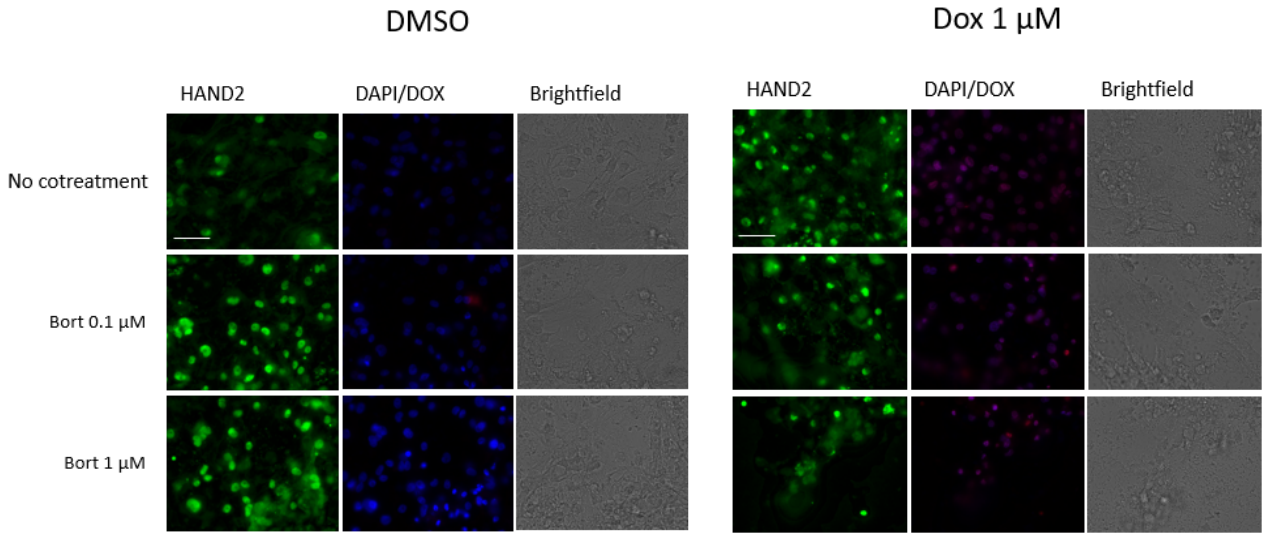
(g)

Figure 8: The top figures show microscopic images of different signals; the IRX4, HAND2, DAPI/DOX and brightfield signals. These images are made after treatment with anthracyclines and with or without cotreatment with Bortezomib. The cells were treated with 0 µM (DMSO) (a), 1 µM (b), 5 µM (c) and 10 µM (d) of Doxorubicin and 5 µM of Amrubicin (e) and Aclarubicin (f). For the cotreatment a concentration of 1 µM was used. The scale bar is the same for every picture and has a length of 100 µm. The quantification of the HAND2 results is shown in (g).

To check the results of the executed Bortezomib cotreatment shown in figure 8, figure 9 shows results from a different experiment were 0.1 μM and 1 μM Bortezomib cotreatment was added for half an hour, this cotreatment was followed by 24 hours of treatment with anthracyclines.

In agreement with previous experiments, figure 9 shows a dose dependent decrease of HAND2. Figure 9a shows a very low intensity of HAND2 in the untreated cells without cotreatment. The DMSO conditions with cotreatment showed a higher intensity of HAND2. Figures 9b to 9d and 9f show that the addition of a Bortezomib cotreatment does not prevent reduction of the HAND2 expression caused by anthracycline treatment. There does seem to be an increase of HAND2 expression when cotreatment is added to a 5 μM and 10 μM of Doxorubicin treatment. However, there also seems to be a decrease of HAND2 expression when cotreatment is added to a 5 μM of Aclarubicin treatment. Similar to previous results, treatment with Amrubicin shows a strong signal in figure 9c. There does not seem to be a difference when cotreatment is added.

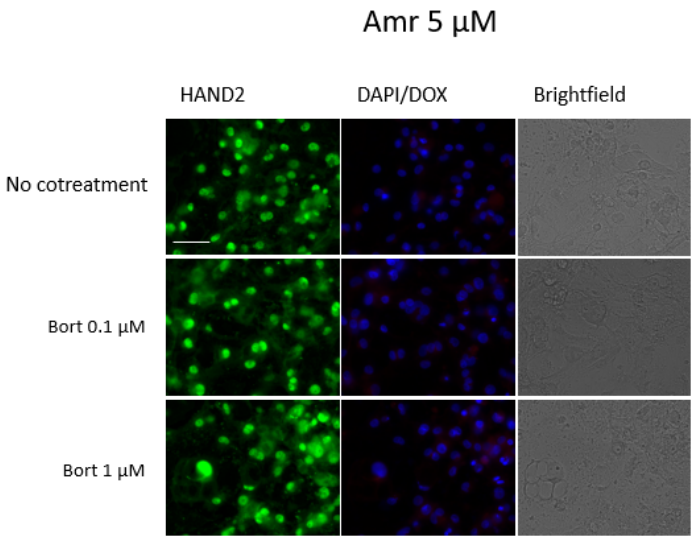
The quantification shown in figure 9g shows a decrease of HAND2 expression with an increasing concentration of Doxorubicin treatment. When cotreatment is added the HAND2 expression seems to increase after treatment with 5 μM and 10 μM of Doxorubicin and decrease after treatment with 5 μM of Aclarubicin.


DMSO
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 0.1 µM
Bort 1 µM

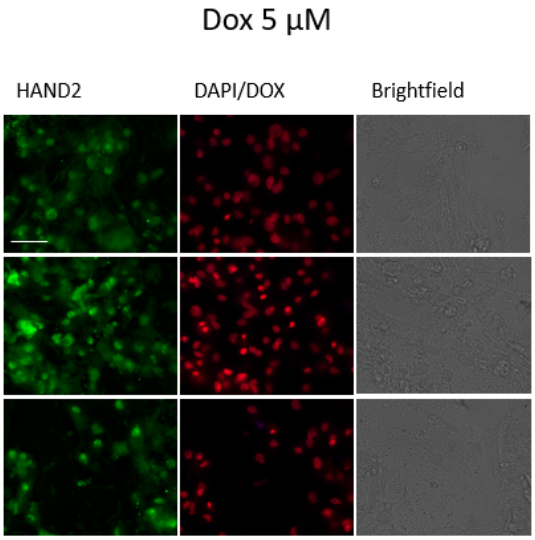
(a)

Dox 1 µM
HAND2
DAPI/DOX
Brightfield

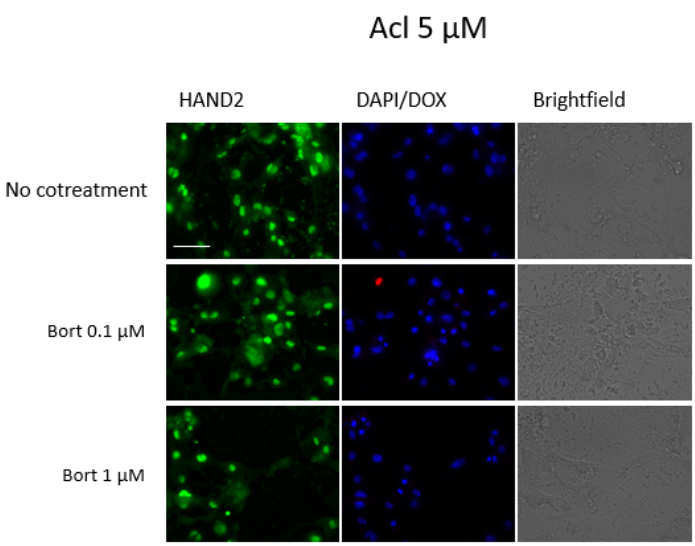
(b)


Amr 5 µM
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 0.1 µM
Bort 1 µM

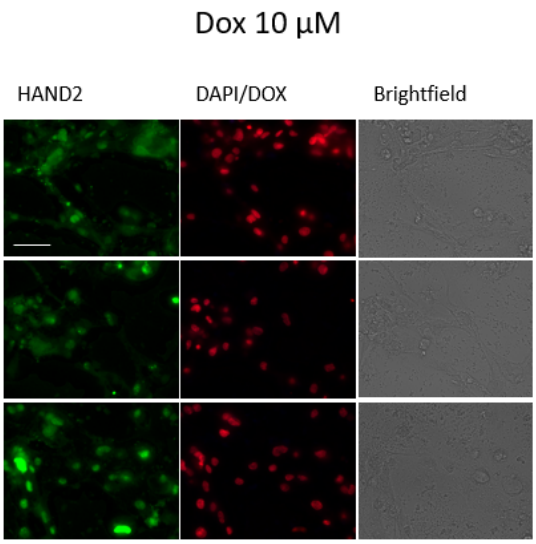
(c)


Dox 5 µM
HAND2
DAPI/DOX
Brightfield

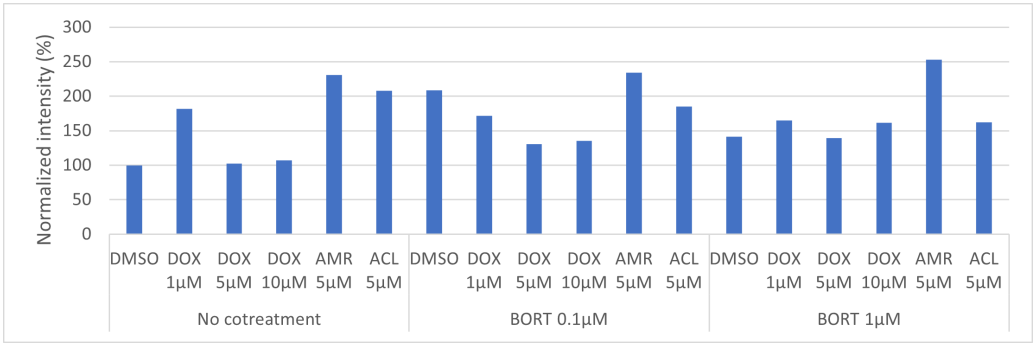
(d)


Acl 5 µM
HAND2
DAPI/DOX
Brightfield
No cotreatment
Bort 0.1 µM
Bort 1 µM

(e)


Dox 10 µM
HAND2
DAPI/DOX
Brightfield

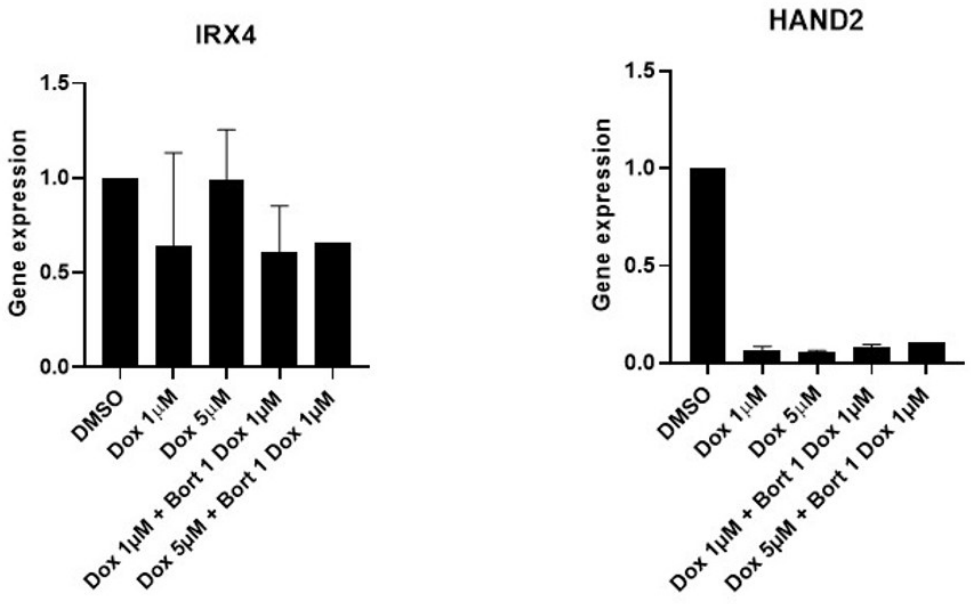
(f)



(g)

Figure 9: The top figures show microscopic images of different signals; the HAND2, DAPI/DOX and brightfield signals. These images are made after treatment with an anthracycline and with or without cotreatment with Bortezomib. The cells were treated with 0 μM (DMSO) (a), 1 μM (b), 5 μM (d) or 10 μM (f) of Doxorubicin and 5 μM of Amrubicin (c) and Aclarubicin (e). For the cotreatment a concentration of 0.1 μM and 1 μM of Bortezomib was used. The scale bar is the same for every picture and has a length of 100 μm. The quantification of the HAND2 results is shown in (g).

To determine if the found effect of Bortezomib on HAND2 protein expression also applies to its gene expression, cotreatment was added to the qPCR. IPSC-cardiomyocytes were first treated with 1 μM Bortezomib for half an hour and then treated with 1 μM or 5 μM Doxorubicin for 24 hours. The cardiomyocytes treated with cotreatment show no change in gene expression of IRX4 compared to the cardiomyocytes without cotreatment. Figure 10b shows that Bortezomib was not able to prevent the downregulation of the HAND2 gene expression.



(a) *(b)*

Figure 10: The graphs show the gene expression of IRX4 (a) and HAND2 (b) after treatment with Doxorubicin and with or without cotreatment with Bortezomib. The cells were treated with 0 μM (DMSO), 1 μM, 5 μM and 10 μM of Doxorubicin. For the cotreatment a concentration of 1 μM and 5 μM of Bortezomib was used.

4 Discussion

The aim of this research is to find an answer to the following research questions:

- *How do anthracyclines affect the gene and protein expression of the transcription factors IRX4 and HAND2 in cardiomyocytes?*
- *How does Bortezomib affect the gene and protein expression of the transcription factors IRX4 and HAND2 in cardiomyocytes after treatment with anthracyclines?*

The graph shown in figure 4a shows that the gene expression of IRX4 is not affected by Doxorubicin. The error bars of the Doxorubicin-treated conditions overlap with each other and with the DMSO condition.

When staining the cells for IRX4, it was expected that this signal would be detected in the nucleus. However, in the results the microscopic images show otherwise. The signal is mostly visible in the cytosol of the cells. This can be explained with literature. An experiment done by Nelson et al. showed that in mice cardiovascular stem cells IRX4 can be found in the cytosol until postnatal day 4 [15]. Between postnatal day 5 and 6 IRX4 will translocate to the nucleus. If this is also the case in human cells, this can explain the location of IRX4 in this research. The hPSC-cardiomyocytes used, do not have aged enough for IRX4 to translocate to the nucleus. These cells are not as mature as postnatal cells [16] and so the staining of IRX4 is found in the cytosol instead of in the nucleus.

When looking at figure 5 and figure 6, it can be seen that the IRX4 images show no change in intensity of the IRX4 protein signal after addition of any anthracycline and over time.

In contrast to no or a low concentration of Doxorubicin treatment in the wash out experiment (figure 7) a high concentration of Doxorubicin shows a decrease in IRX4 expression. However, when looking at the brightfield images it can be seen that these cells are no longer viable. The decrease in IRX4 signal can be explained because dying or dead cells show an increased autofluorescence signal [17][18]. Because most autofluorescence is detected at a shorter wavelength than 647 nm, the images show very little signal.

Other striking results from the wash out experiment are the results after 14 days of treatment with 1 μM of Doxorubicin. There is no signal visible in the IRX4 and DAPI/DOX images. The reason for this is that the signal was so weak that after processing the images there was no signal at all. This very weak signal is the result of a bacterial contamination.

Aside from these unexpected results, the wash out experiment shows no change in IRX4 protein expression over time. This experiment also shows that it is not useful to perform a long timelapse experiment with high concentrations of Doxorubicin because the cardiomyocytes will not survive it.

Figure 8 shows cells treated with anthracyclines and with or without a Bortezomib cotreatment. However, the IRX4 results from the treated cells without cotreatment show different results than shown before. Contrary to previous results treatment with 5 μM and 10 μM increases the IRX4 expression. The cotreatment does not seem to have an effect on the protein expression of IRX4. Since the timelapse and wash out did not show a change in IRX4 protein expression, it is not expected that Bortezomib cotreatment has any effect. The hypothesis for Bortezomib was to prevent knock-down of the transcription factors but since IRX4 is not knocked-down Bortezomib should not have any effects on the IRX4 protein expression.

Figure 10a shows no effect of Doxorubicin or Bortezomib on the IRX4 gene expression. This means treatment with Doxorubicin for 24 hours and adding a Bortezomib cotreatment do not downregulate the IRX4 gene.

HAND2 shows a decrease in gene expression after treatment with Doxorubicin (figure 4b. This corresponds with the expectation and literature. Earlier research showed that the expression of HAND2 reduces by 85% after 48h of exposure to 1 μM of Doxorubicin [9]. These conditions are not exactly equal but the results correspond with expectations based on this earlier research.

In all immunohistochemistry experiments (figures 5 to 9), the DMSO conditions show a very low signal for HAND2 expression. Explanation of this could be that not all cells on the plates were fixated at the same time. This means that the unfixated cells had already been in contact with evaporated formaldehyde before they were fixated themselves. This might have led to partly fixation. This might explain the 'increases' in protein expression after treatment with anthracyclines. The anthracycline-treated cells most likely do not have increased expression but the low expression in the DMSO conditions should have been higher than in anthracycline-treated cells, which would give a decrease in expression after treatment. For next experiments the different timeconditions should be placed on different plates so cells will not get partly fixated before fixation.

HAND2 protein expression decreases over time after Doxorubicin treatment. This can be seen in figure 5. Treatment for a maximum of 8 hours shows a decreasing expression of HAND2. This expression also decreases with a higher concentration of Doxorubicin.

Figure 5d shows that after treatment with 5 μM and 10 μM of Doxorubicin the intensity of the HAND2 signal decreases over time. This result is less clear from the 1 μM treatment. It shows a reduction of intensity after 8 hours of treatment with 1 μM of Doxorubicin. After 6 hours of treatment there seems to be more protein expression. The deviation of this condition is widely spread. This conditions should be further researched to conclude if treatment with 1 μM of Doxorubicin also causes this decrease in protein expression.

After 24 hours of treatment with different anthracyclines, figure 6 shows an increase of HAND2 protein expression with increasing anthracycline concentrations. However, before it was explained how different timeconditions were placed on one plate. This was also the case for this experiment. The cells treated for 24 hours were on the same plate as the cells treated for 0 to 8 hours. This might explain the unexpected results and because of this to be able to draw any conclusions from this experiment, it should be repeated on a separate plate from the 0-8h experiment.

In figures 6, 8 and 9 Amrubicin shows a very strong signal in the GFP channel. This can be explained because Amrubicin most likely gives an autofluorescence signal in the GFP channel. Because of this, it looks like there is a lot of HAND2 signal but this is not necessarily the case. Literature shows that Amrubicin causes limited cardiotoxicity compared to Doxorubicin and Aclarubicin [1]. To determine the effect of Amrubicin treatment on the HAND2 expression, these experiments should be repeated for Amrubicin but with a Cy5 secondary antibody for the HAND2 staining.

Before it was mentioned that when treated with high concentration of Doxorubicin, cells do not survive. The wash out experiment showed a very strong signal in the GFP channel after treatment with 5 μM and 10 μM of Doxorubicin. This strong signal is caused by autofluorescence signal of the dead or dying cells [17][18]. Because of this increase in autofluorescence, it looks like there are more HAND2 protein present in these conditions. However, at a wavelength of 488 nm a lot of autofluorescence is detected [18]. This means the signal visible in these images is not caused by HAND2 protein but by the autofluorescence from the dead cardiomyocytes.

Like mentioned for IRX4, 14 days after treatment with 1 μM shows no or a weak signal in all channels. The reason for this is a bacterial contamination.

Figure 9 shows a decrease of HAND2 in cells treated with 1 μM, 5 μM and 10 μM of

Doxorubicin. However, there does not seem to be a big difference in protein expression between untreated cells and cells treated with Aclarubicin. As mentioned before DMSO shows a very low signal. Looking at the DMSO with 0.1 μM of Bortezomib cotreatment, this looks more like the signal intensity expected from DMSO. Assuming addition of 0.1 μM Bortezomib does not have an effect on the HAND2 expression in DMSO, this condition can be used as control condition. In this case, treatment with Doxorubicin clearly shows a dose-dependent reduction of the HAND2 protein expression and also Aclarubicin-treated cells show a decrease in HAND2 expression.

To determine whether the reduction of HAND2 can be prevented, Bortezomib was added as cotreatment (figures 8 and 9). Even though figure 8 does not show the effect of anthracycline treatment as expected and shown in figures 5 and 9, it can be concluded that the addition of Bortezomib cotreatment does not prevent HAND2 reduction. Figure 9 shows an increasing HAND2 expression after 5 μM and 10 μM of Doxorubicin treatment and cotreatment, but a decreasing HAND2 expression after 5 μM of Aclarubicin treatment and cotreatment. This experiment also shows that with cotreatment, Doxorubicin still reduces HAND2 expression in a dose-dependent way. Based on these two Bortezomib cotreatment experiments, it can be concluded that a cotreatment with Bortezomib does not prevent the reduction of HAND2 in cardiomyocytes.

When looking at figure 10b it can be seen that the bars with and without cotreatment are very low and similar. This means that a cotreatment with Bortezomib is not able to prevent the reduction of HAND2 gene expression. Since the bars with and without treatment are so similar, from this graph it can not be seen if the error bars overlap. To make sure Bortezomib does not change the gene expression of HAND2 a statistical analysis is needed.

Out of all of these results it can be concluded that IRX4 is not affected by anthracyclines on gene level and neither on protein level. The HAND2 gene expression on the other hand seems to be highly affected by anthracyclines. This is also the case on protein level. Both the gene and protein expression show a decrease after treatment with anthracyclines. No conclusion can be drawn about the effects of Amrubicin treatment on HAND2 protein expression based on the results of this research because of autofluorescence signal in the GFP channel. This research also shows that Bortezomib does not have an effect on the gene and protein expression of IRX4 and is not able to prevent reduction of gene and protein expression of HAND2.

The effects of anthracyclines on the IRX4 protein expression can possibly be explained by the location where these proteins were found. IRX4 proteins were found in the cytosol instead of the nucleus. However, the pathways of altered gene expression caused by Doxorubicin (as explained in the introduction) take place in the nucleus. This could explain why anthracyclines do not affect IRX4 protein expression but do reduce HAND2 protein expression. Based on this hypothesis, this research cannot conclude if anthracycline treatment has the same effects on IRX4 expression in clinic as shown in this research. Because the HAND2 and IRX4 proteins are not found at the same location in the cell, it is not known if the knock-down of transcription factors is a selective process or not. However, the gene expression of HAND2 is reduced by Doxorubicin treatment while the IRX4 gene expression is not, it can be expected that the knock-down of transcription factors is a selective process because both genes can be found in the nucleus. To make sure this process is selective and to further understand it, more research should be done.

In the introduction the effects of depletion of HAND2 are mentioned. Based on this information, it can be concluded that treatment with Doxorubicin and Aclarubicin can cause hypoplasia of the heart. Because of this, Doxorubicin and Aclarubicin cannot be recommended for clinical use. Since Bortezomib did not show the possibility to prevent downregulation of HAND2, this also cannot be recommended for clinical use.

5 Conclusion

Based on the results and the discussion, it can be concluded that on gene and protein level IRX4 is not affected by the anthracyclines; Doxorubicin, Amrubicin and Aclarubicin. HAND2 on the other hand, shows a decrease in gene expression after treatment with Doxorubicin and Aclarubicin. Also the HAND2 protein expression seems to be affected by anthracyclines. The HAND2 protein expression shows increasing decrease when the concentration of Doxorubicin increases and also when the treatment time increases. No conclusions can be drawn about the effects of Amrubicin treatment on HAND2 protein expression based on this research because of autofluorescence signal in the GFP channel.

This research also shows that Bortezoib does not have an effect on the gene and protein expression of IRX4 and is not able to prevent reduction of gene and protein expression of HAND2.

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