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ORIGINAL RESEARCH

Evaluation of Senescence and Its Prevention in Doxorubicin-Induced Cardiotoxicity Using Dynamic Engineered Heart Tissues

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ABSTRACT

BACKGROUND Doxorubicin is an essential cancer treatment, but its usefulness is hampered by the occurrence of cardiotoxicity. Nevertheless, the pathophysiology underlying doxorubicin-induced cardiotoxicity and the respective molecular mechanisms are poorly understood. Recent studies have suggested involvement of cellular senescence.

OBJECTIVES The aims of this study were to establish whether senescence is present in patients with doxorubicin-induced cardiotoxicity and to investigate if this could be used as a potential treatment target.

METHODS Biopsies from the left ventricles of patients with severe doxorubicin-induced cardiotoxicity were compared with control samples. Additionally, senescence-associated mechanisms were characterized in 3-dimensional dynamic engineered heart tissues (dyn-EHTs) and human pluripotent stem cell-derived cardiomyocytes. These were exposed to multiple, clinically relevant doses of doxorubicin to recapitulate patient treatment regimens. To prevent senescence, dyn-EHTs were cotreated with the senomorphic drugs 5-aminoimidazole-4-carboxamide ribonucleotide and resveratrol.

RESULTS Senescence-related markers were significantly up-regulated in the left ventricles of patients with doxorubicin-induced cardiotoxicity. Treatment of dyn-EHTs resulted in up-regulation of similar senescence markers as seen in the patients, accompanied by tissue dilatation, decreased force generation, and increased troponin release. Treatment with senomorphic drugs led to decreased expression of senescence-associated markers, but this was not accompanied by improved function.

CONCLUSIONS Senescence was observed in the hearts of patients with severe doxorubicin-induced cardiotoxicity, and this phenotype can be modeled in vitro by exposing dyn-EHTs to repeated clinically relevant doses of doxorubicin. The senomorphic drugs 5-aminoimidazole-4-carboxamide ribonucleotide and resveratrol prevent senescence but do not result in functional improvements. These findings suggest that preventing senescence by using a senomorphic during doxorubicin administration might not prevent cardiotoxicity. (J Am Coll Cardiol CardioOnc 2023;5:298-315) © 2023 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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Doxorubicin is one of the most effective chemotherapeutic drugs. However, its therapeutic use is limited by its tendency to cause cardiotoxicity, which manifests clinically as cardiomyopathy with a decreased ejection fraction and arrhythmias.[1] Although doxorubicin has been administered to patients for decades, the mechanisms explaining the pathogenesis of this side effect are not fully understood.[1] Circulating doxorubicin levels have been measured in patients and fall in the range of 0.025 to 0.250 μmol/L.[2-4] However, often much higher concentrations of doxorubicin (>5 μmol/L) are used in experimental setups.[5,6] The relevance of these differences in concentration was demonstrated by Maejima et al,[7] who showed that high concentrations of doxorubicin induce cell death, while lower concentrations induce a milder phenotype, termed senescence.[8] Induction of senescence upon doxorubicin treatment has been shown in human cancer cells[9] but is not yet linked to cardiotoxicity.

The definition of senescence in cardiomyocytes remains elusive, as these cells are already in a state of irreversible cell-cycle arrest. Senescent cardiomyocytes present a series of functional declines, including contractile and mitochondrial dysfunction, and expression of molecular markers such as p16 and p21.[10] The impact of senescent cells within the heart also remains controversial. Senescent cardiomyocytes show reduced energy metabolism and impaired contractility, reducing cardiac output. However, removal of senescent cells from aging hearts using senolytic drugs has shown conflicting results.[11] Recently it was shown in mice that the combination of dasatinib and quercetin, which reduces senescent cell abundance in aged mice, was associated with improved left ventricular function, but side effects included hematologic dysfunction, fluid retention, and QT interval prolongation.[11] Although senescence has been associated with doxorubicin-induced cardiotoxicity in an experimental setting, it remains unclear whether this plays a role in developing cardiotoxicity in patients with cancer.

Senomorphic agents are a class of drugs that suppress markers of senescence, in particular the senescence-associated secretory phenotype (SASP), or their secretory phenotype without inducing apoptosis. Promising results in prevention of cardiotoxicity have been obtained with this class of drugs.[12,13]

In this study we therefore assessed the presence of senescent cells in heart tissue from patients with severe doxorubicin-induced cardiotoxicity. Three-dimensional dynamic engineered heart tissues (dyn-EHTs) were applied to model this condition in vitro by the clinically relevant administration of repeated low doses of doxorubicin. Subsequently, we assessed the therapeutic potential of senomorphic drugs to alleviate the burden of senescence in dyn-EHTs treated with doxorubicin.

ABBREVIATIONS AND ACRONYMS

AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide

dyn-EHT = dynamic engineered heart tissue

FC = fold change

hPSC-CM = human pluripotent stem cell-derived cardiomyocyte

mRNA = messenger RNA

SASP = senescence-associated secretory phenotype

METHODS

Detailed methods, including cell culture, cardiac differentiation,[14] dyn-EHT generation,[15] and all assays and laboratory methods, can be found in the Supplemental Appendix and Supplemental Tables 1 to 3.

HUMAN HEART SAMPLES. Heart tissue from 7 patients with doxorubicin-induced cardiomyopathy was obtained during left ventricular assist device implantation. These samples were compared with healthy tissue from 5 donor hearts that could not be transplanted for logistic reasons. The study met the criteria of the code of proper use of human tissue in the Netherlands. The scientific advisory board approved collection of the human heart tissue of the biobank of the University Medical Center Utrecht

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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TABLE 1 Patient Characteristics

	Age, y[a]	Gender	EF (%)	Cumulative Doxorubicin Dose, mg/m^2	Cancer Type	Other Treatments
Control 1	50	Male	NA	NA	NA	NA
Control 2	51	Male	NA	NA	NA	NA
Control 3	40	Female	NA	NA	NA	NA
Control 4	20	Male	NA	NA	NA	NA
Control 5	51	Male	NA	NA	NA	NA
Patient 1	26	Male	10	240	Ewing sarcoma	Radiotherapy, etoposide, vincristine, actinomycin D, ifosfamide
Patient 2	43	Male	12	400	B cell lymphoma	Radiotherapy, rituximab, cyclophosphamide, vincristine, prednisolone
Patient 3	56	Female	20	300	Breast cancer	Radiotherapy, docetaxel, cyclophosphamide
Patient 4	63	Female	10	360	Breast cancer	Radiotherapy, cyclophosphamide
Patient 5	63	Male	12	400	B cell lymphoma	Rituximab, cyclophosphamide, vincristine, prednisolone
Patient 6	40	Male	25	540	Rhabdomyosarcoma	Cyclophosphamide, dactinomycin, vincristine
Patient 7	57	Female	26	240	Breast cancer	Radiotherapy, cyclophosphamide, docetaxel

[a]Age at left ventricular assist device implantation, heart transplantation, or collection upon death.
EF = ejection fraction; NA = not applicable.

(protocols 12/387 and 15/252). Written informed consent was obtained or, in certain cases, waived by the ethics committee when obtaining informed consent was not possible because of the death of the patient. The study complied with the Declaration of Helsinki.

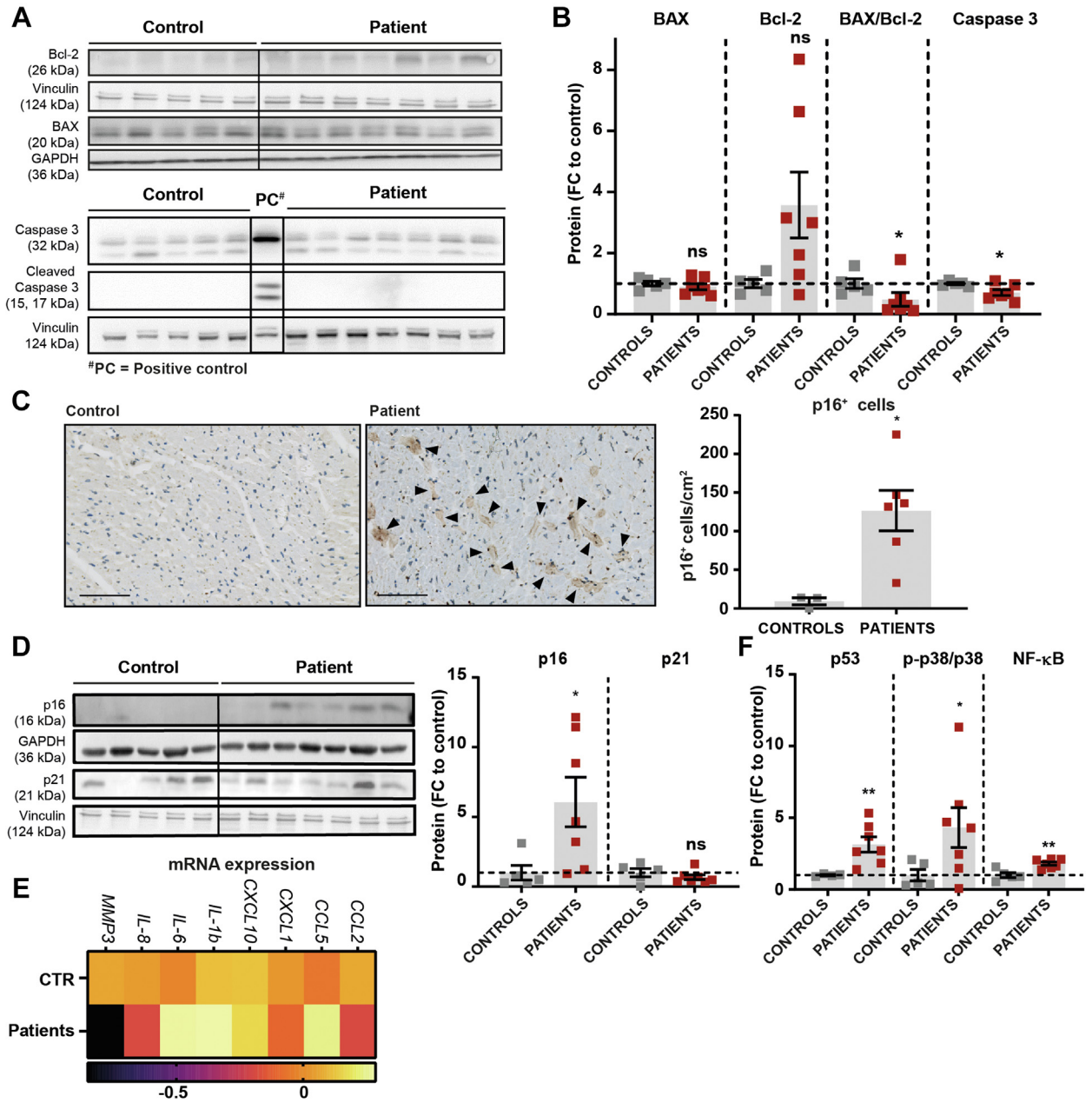
STATISTICAL ANALYSIS. Continuous data are presented as mean ± SEM or mean ± SD and represent individual values, sample means, or means of ≥3 independently performed experiments. For several parameters, fold changes (FCs) against control or baseline were calculated, which is specified in the figure legends. For some messenger RNA (mRNA) expression heatmaps, log FC was used for better visualization, which is specified in the figure legends. Differences were assessed using Student's *t*-test or 1-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple pairwise comparisons. Repeated-measures ANOVA was used to determine the statistical significance of the dyn-EHT functional parameters and was carried out using the *P* values displayed in Supplemental Table 3. The repeated-measures ANOVA was performed using the rstatix package for R (R Foundation for Statistical Computing). Tissue groups (control, 4-hit [described later], control + resveratrol/5-aminoimidazole-4-carboxamide ribonucleotide [AICAR], and 4-hit + resveratrol/AICAR) and day of measurement were used as fixed effects. The assumption of sphericity was evaluated using Mauchly's test for sphericity. Further testing was done depending on the outcome. When the variable "day" resulted in a statistically significant *P* value, a 1-way ANOVA was done, followed by the Bonferroni post hoc test for multiple pairwise comparisons. When the variable "group" resulted in a statistically significant *P* value, a pairwise independent *t*-test was done. These analyses were done in R version 4.2.0. For all tests, *P* values <0.05 were considered to indicate statistical significance.

RESULTS

PATIENT CHARACTERISTICS. Cardiac samples from 7 patients with severe doxorubicin-induced heart failure were collected during left ventricular assist device implantation (Table 1). The mean ± SD age of these patients was 49.7 ± 13.8 years, cumulative doxorubicin dose was 354 ± 106 mg/m^2, and left ventricular ejection fraction was 16% ± 7%. The group consisted of 4 men and 3 women. The donors of the control cardiac samples had a mean age of 42.4 ± 13.3 years and included 4 men and 1 woman.

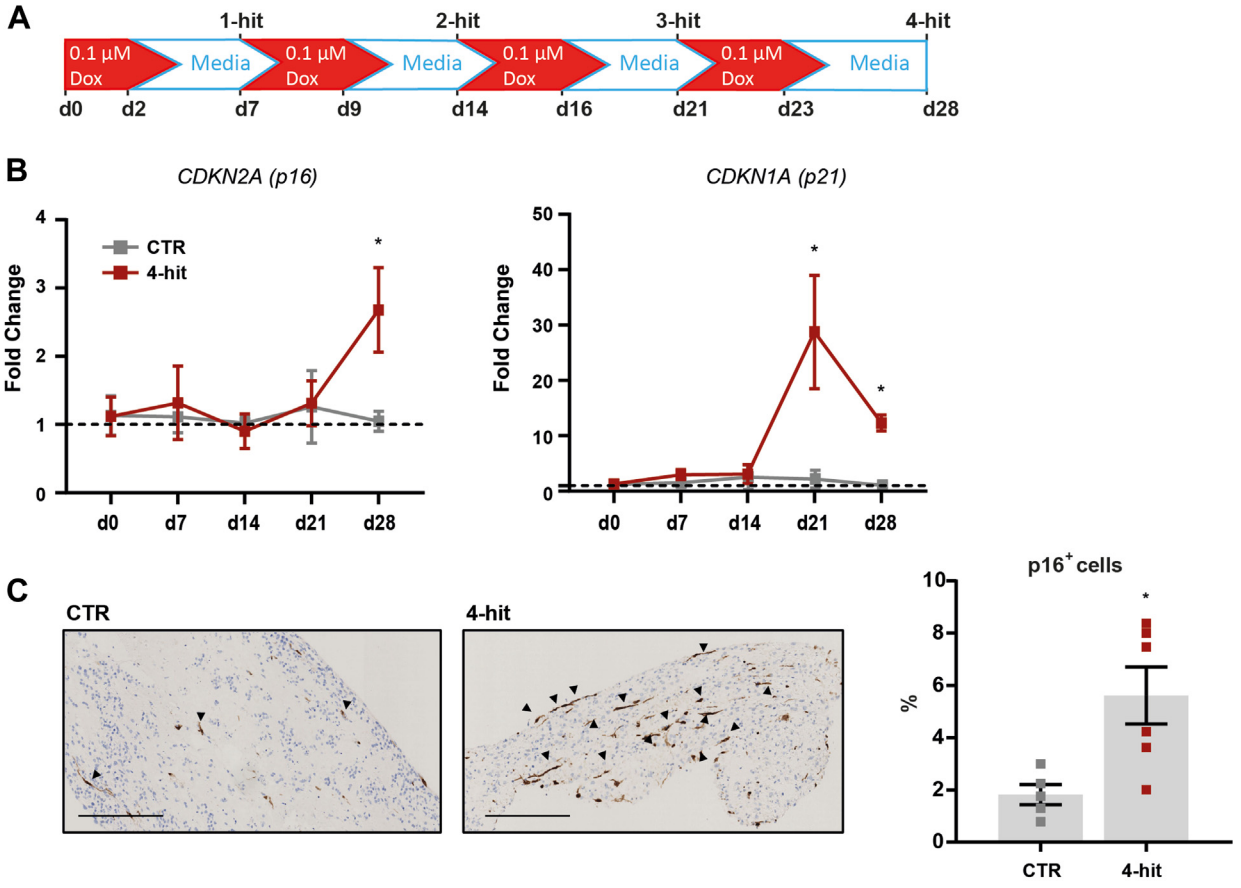
SENESCENCE IS INDUCED IN PATIENTS WITH SEVERE CARDIOTOXICITY. Doxorubicin-induced damage was assessed by evaluating 2 major pathways: apoptosis and senescence. No evidence for the activation of apoptotic pathways was found in these samples, as BAX/Bcl-2 ratio and caspase-3 levels were decreased

FIGURE 1 **Senescence Is Present in the Hearts of Patients With Doxorubicin-Induced Cardiotoxicity**



Gene expression of several senescence and apoptosis markers was assessed in patient hearts. No changes in apoptosis genes were observed, while senescence genes were up-regulated. **(A)** Western blot (WB) images of BAX, Bcl-2, caspase-3, and cleaved caspase-3. The positive control for caspase-3 consists of human pluripotent stem cell–derived cardiomyocytes treated with 5 μM doxorubicin for 24 hours. **(B)** WB analysis of BAX, Bcl-2, and caspase-3 expression. **(C)** Immunohistochemical p16 staining of control and patient cardiac tissue. p16-positive cells are indicated with **black arrows**. Scale bar: 100 μm. Quantification is normalized by tissue area. **(D)** WB analysis of p16, p21, **(E)** p53, phosphorylated p38MAPK (against total p38MAPK), and NF-κB expression in control and cardiotoxic hearts. **(F)** Messenger RNA (mRNA) expression of senescence-associated secretory phenotype factors in control subjects and patients (log-transformed fold changes [FCs]). Bar graphs show mean ± SEM and individual values. The heatmap shows means. *$P < 0.05$ and **$P < 0.01$. Statistical significance was determined using Student's *t*-test, except for **(B)**. Because of an outlier, the Mann-Whitney *U* test was used. CTR = control; ns = not significant.

FIGURE 2 p16 and p21 Follow Predicted Expression Patters in Senescent Dyn-EHTs



Dynamic engineered heart tissues (dyn-EHTs) were exposed to multiple low doses of doxorubicin (Dox). Increases of CDKN2A (p16) and CDKN1A (p21) over time were observed. **(A)** Treatment scheme for the dyn-EHTs. Forty-eight hours of 0.1 μmol/L doxorubicin was followed by 5 days in fresh media, repeated 4 times (4-hit). **(B)** mRNA expression of senescence markers CDKN2A (p16) and CDKN1A (p21) over time. **(C)** Immunohistochemical p16 staining of CTR and 4-hit-treated tissues. p16-positive cells are indicated with **black arrows**. Scale bar: 300 μm. Linear graphs show mean ± SEM. Bar graphs show mean ± SEM and individual values. *$P < 0.05$. Statistical significance was determined using Student's *t*-test or 1-way analysis of variance with the Bonferroni post hoc test for multiple pairwise comparisons. Abbreviations as in Figure 1.

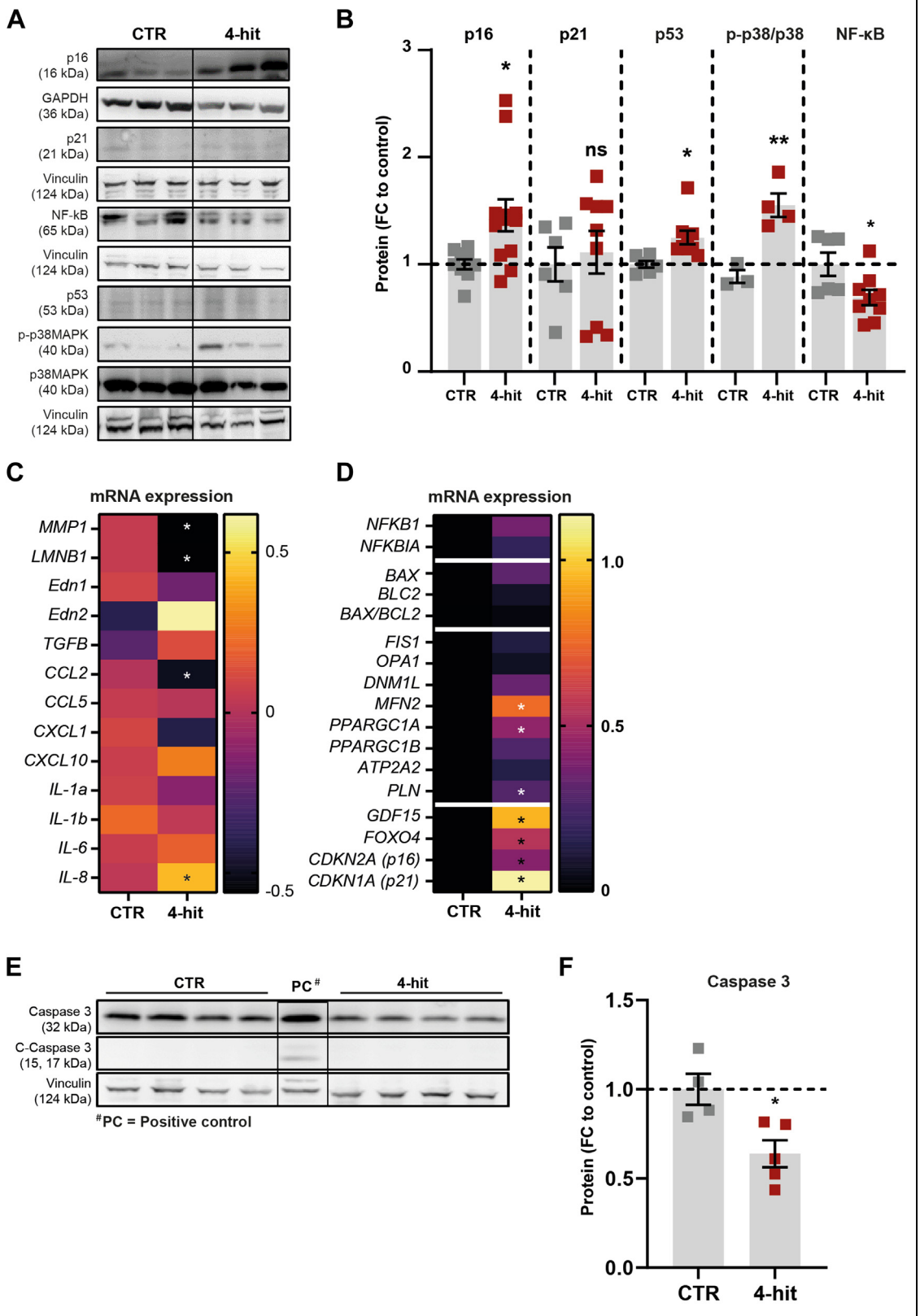
($P = 0.048$ and $P = 0.029$, respectively) compared with control (Figures 1A and 1B, Supplemental Figure 1A). Induction of senescence among the cardiomyocytes in the human heart upon doxorubicin treatment was explored by counting $p16^+$ cardiomyocytes, as described in the Supplemental Appendix. A 14-fold increase of $p16^+$ cardiomyocytes (mean ± SD 9.3 ± 8.0 vs 126.6 ± 64.18 $p16^+$ cells/cm^2; $P = 0.01$) was observed in patient tissues compared with those from control subjects (Figure 1C). Western blot substantiated these findings, and a 6-fold increase ($P = 0.043$) in p16 protein levels was found (Figure 1D).

Expression of p21, a marker of acute senescence,[16] did not differ between patient and control hearts (Figure 1D). Further analysis showed that expression of proteins otherwise involved in senescence was increased, such as p53 (FC = 3.1; $P = 0.015$), phosphorylated p38MAPK (FC = 4.3; $P = 0.039$), and NF-κB (FC = 1.8; $P = 0.002$) (Figure 1E, Supplemental Figure 1A). Similarly, the downstream remodeling factors MMP2 (FC = 1.77; $P = 0.026$), MMP9 (FC = 5.35; $P = 0.031$), and TIMP2 (FC = 4.60; $P = 0.005$) showed increased expression (Supplemental Figures 1A and 1B).

No significant differences could be found in the mRNA expression of several SASP factors (Figure 1F).

Because of the involvement of mitochondria in senescence,[17] biogenesis, fission, and fusion of these

FIGURE 3 **Up-Regulation of Senescent Markers**



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organelles were assessed. No changes were found in gene expression related to oxidative phosphorylation, but expression of *MFN2* (FC = 1.5; $P = 0.034$), *PPARGC1A* (FC = 1.1; $P = 0.050$), and *PPARGC1B* (FC = 1.1; $P = 0.050$) was increased (Supplemental Figures 1C and 1D).

ESTABLISHING A HUMAN DYN-EHT SYSTEM TO MODEL DOXORUBICIN-INDUCED TOXICITY. To model doxorubicin-induced cardiotoxicity, dyn-EHTs were used.[15] Dyn-EHTs were subjected to doxorubicin in a manner that reflects the treatment scheme in the clinical setting[2-4]: 0.1 μmol/L doxorubicin was added to the medium for 48 hours, after which the medium was changed and left for 5 days. This was repeated 4 times (referred to as "4-hit") (**Figure 2A**).

mRNA expression of the senescence markers *CDKN2A* (p16), *CDKN1A* (p21), *GDF15*, and *FOXO4* was assessed at baseline and on days 7, 14, 21, and 28 of treatment. *CDKN2A* (p16) expression increased from day 14, reaching significance after 4 doses (FC = 2.7; $P = 0.018$), while *CDKN1A* (p21) showed an increase at day 21 and a subsequent decrease (day 28) (**Figure 2B**). *GDF15* (FC = 6.5; $P = 0.009$) and *FOXO4* (FC = 2.2; $P = 0.018$) showed gradual increases over time ($P = 0.015$ and $P = 0.032$, respectively) (Supplemental Figure 2A). The increase of p16 was also shown on protein level through staining (FC = 4.8; $P = 0.014$) and western blot (FC = 1.5; $P = 0.018$) (**Figures 2C, 3A, and 3B**). p21 protein expression did not increase significantly with treatment (**Figures 3A and 3B**). Besides, p53 levels increased (FC = 1.3; $P = 0.005$), phosphorylated p38MAPK levels increased (FC = 1.6; $P = 0.003$), and NF-κB levels decreased (FC = 0.7; $P = 0.026$) (**Figures 3A and 3B**). mRNA expression of several SASP factors was explored, and IL-8 showed a significant increase (FC = 3.0; $P = 0.008$) (**Figure 3C**).

mRNA expression in fibroblasts showed up-regulation of p21 expression (FC = 3.58; $P = 0.032$) but not of p16. Several SASP factors were up-regulated, including GDF-15 (FC = 3.33; $P = 0.018$), MMP1 (FC = 2.53; $P < 0.001$), Edn1 (FC = 4.31; $P < 0.001$), and IL-1b (FC = 2.12; $P < 0.001$). CCL2 (FC = 0.67; $P = 0.011$), CXCL1 (FC = 0.48; $P = 0.002$), and IL-6 (FC = 0.56; $P < 0.001$) showed decreased expression (Supplemental Figure 2B).

Absence of cleaved caspase-3 protein and decreased expression of caspase-3 ($P = 0.016$) demonstrated the absence of apoptosis in the 4-hit dyn-EHTs, comparable with the patient heart tissues (**Figures 3D-3F**). These results showed the ability of our dyn-EHTs to model doxorubicin-induced toxicity, mimicking the patient condition.

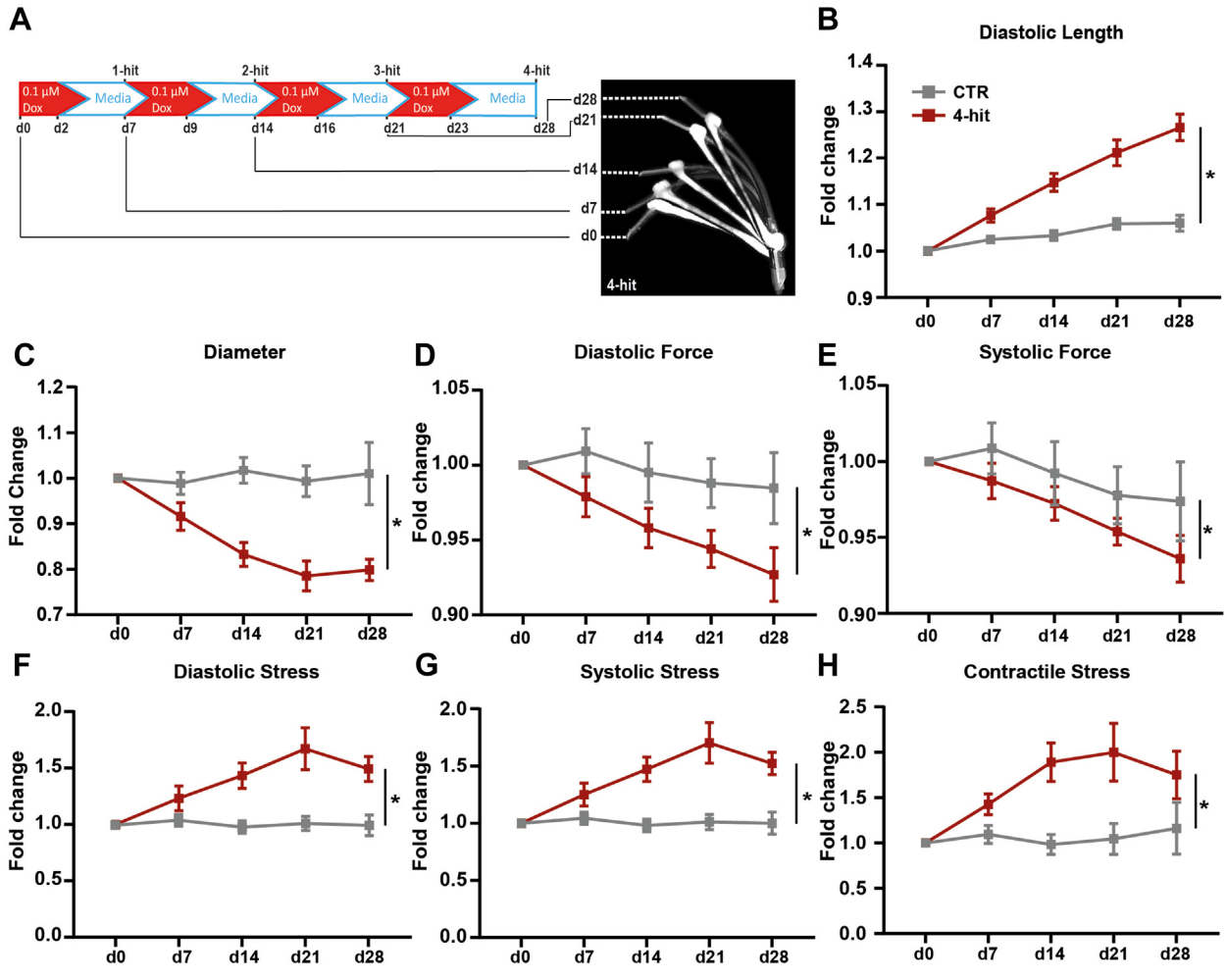
DYN-EHTs TREATED WITH MULTIPLE, RELEVANT DOSES OF DOXORUBICIN DILATE AND SHOW DECREASED FORCE GENERATION. Four-hit dyn-EHTs demonstrated an increase of troponin excretion into the culture medium but did not show damaged sarcomere structures (Supplemental Figures 2D and 2E). N-terminal pro-brain natriuretic peptide release did not increase (Supplemental Figure 2F). On a functional level, we found several parameters affected in 4-hit dyn-EHTs (Videos 1 to 4). At day 28, the diastolic length of 4-hit dyn-EHTs was increased by 15% compared with control (FC = 1.12 vs 1.27; $P = 0.007$), and the diameter decreased by 21% (FC = 1.01 vs 0.8; $P < 0.001$) (**Figures 4A to 4C**). Diastolic and systolic force generation decreased significantly over time during doxorubicin exposure, by −8% and −7%, respectively ($P < 0.001$ for both), whereas this remained stable in control subjects (**Figures 4D and 4E**). Despite this reduction in force, and because of the decrease in tissue diameter, diastolic (preload), systolic, and contractile stress (afterload) increased greatly by day 28, by 50% (FC = 0.99 vs 1.49; $P = 0.008$), 52% (FC = 1.00 vs 1.52; $P = 0.002$), and 80% (FC = 0.95 vs 1.75; $P = 0.03$), respectively (**Figures 4F to 4H**). The contractile shortening did not decline (Supplemental Figure 2G), suggesting a stage of preclinical heart failure, with elevated pre- and afterload and increased cardiomyocyte stress.

DOXORUBICIN TREATMENT IMPAIRED MITOCHONDRIAL FUNCTION IN HUMAN PLURIPOTENT STEM CELL–DERIVED CARDIOMYOCYTES. As markers for mitochondrial biogenesis were up-regulated in patient tissues and dyn-EHTs (**Figure 3D**, Supplemental

FIGURE 3 Continued

To confirm senescence in the 4-hit dyn-EHTs, expression of several markers was assessed, using WB and mRNA expression. **(A)** Representative WBs of p16, p21, p53, phosphorylated p38MAPK (against total p38MAPK), and NF-κB expression and **(B)** quantification. **(C)** mRNA expression of senescence-associated secretory phenotype factors in control and 4-hit dyn-EHTs (log-transformed FCs). **(D)** mRNA expression (log-transformed FC). **(E)** WB analysis of caspase-3 and cleaved caspase-3. The positive control consists of human pluripotent stem cell-derived cardiomyocytes treated with 5 μM doxorubicin for 24 hours. **(F)** Quantification of caspase-3 protein expression. Bar graphs show mean ± SEM and individual values. *$P < 0.05$ and **$P < 0.01$. Statistical significance was determined using Student's *t*-test. Abbreviations as in **Figures 1 and 2**.

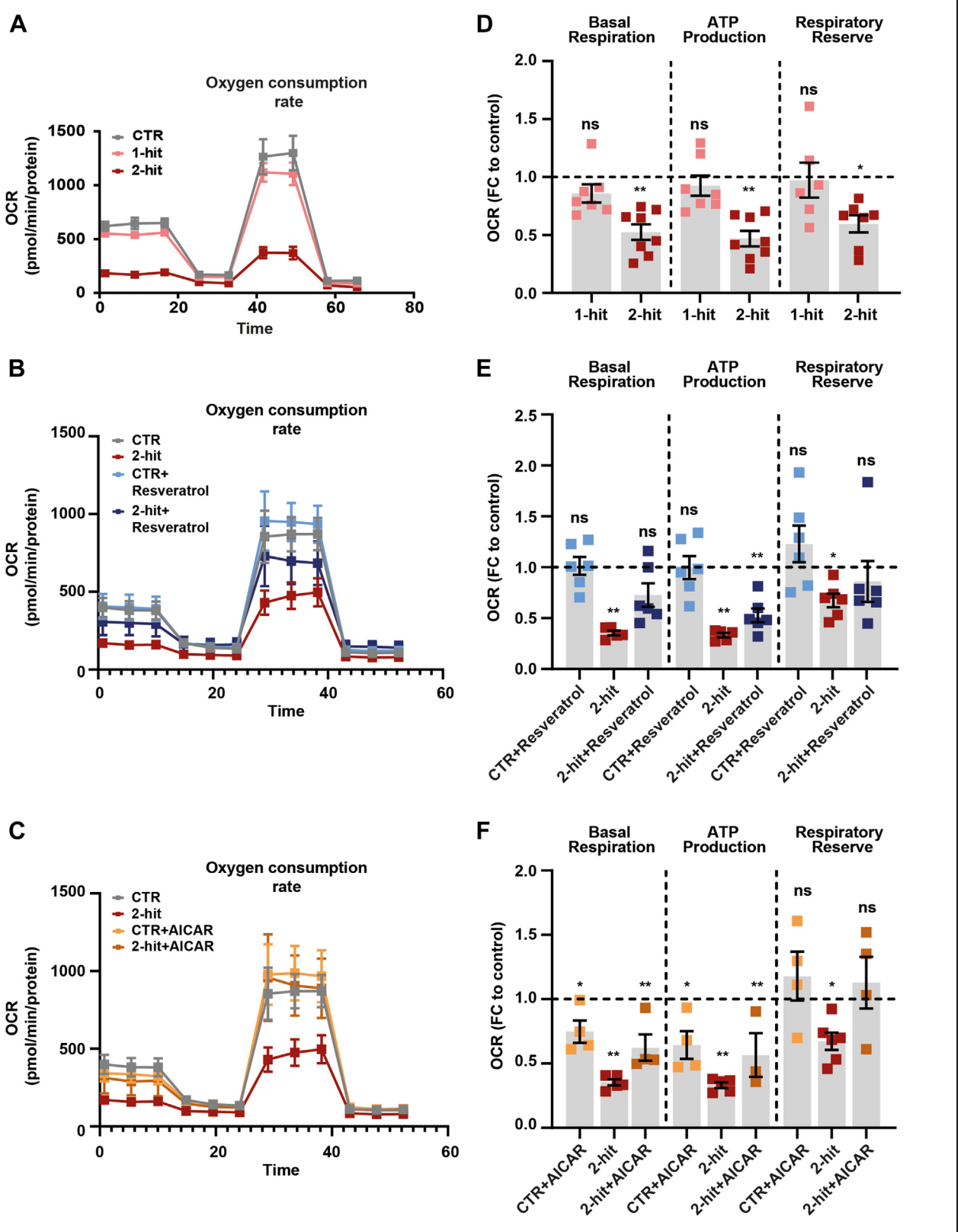
FIGURE 4 Function of Senescent Dyn-EHTs Is Impaired



Senescent dyn-EHTs showed decreased functionality. **(A)** Treatment scheme with elongation of a representative tissue over time. Quantification of diastolic length **(B)**, diameter **(C)**, diastolic force **(D)**, systolic force **(E)**, diastolic stress **(F)**, systolic stress **(G)**, and contractile stress **(H)**. **Gray line** denotes CTR (n = 12); **red line** denotes 4-hit (n = 17). Linear graphs show mean ± SEM. Bar graphs show mean ± SEM and individual values. *$P < 0.05$ and **$P < 0.01$ against control on the same day; $^{\#}P < 0.05$, 4-hit against its own baseline. Statistical significance was determined using 1-way analysis of variance with the Bonferroni post hoc test for multiple pairwise comparisons. Abbreviations as in Figure 2.

Figure 1D), we used a 2-dimensional model based on human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) to examine mitochondrial function. hPSC-CMs were treated with 2 treatments of 0.1 μmol/L doxorubicin for 48 hours, with 48 hours of recovery in between (2-hit). As comparators, cells treated with either 1 low dose (1-hit) or 1 high dose (5 μmol/L) or untreated (control) cells were also included (Supplemental Figure 3A). A single high dose of doxorubicin resulted in apoptosis in the hPSC-CMs, as demonstrated by the presence of cleaved caspase-3 (Supplemental Figures 3B and 3C). Because of these results, 5 μmol/L was not included in further experiments. The 2-hit-exposed hPSC-CMs showed senescence by increased expression of p21 and p53 ($P = 0.031$ and $P = 0.023$, respectively). Furthermore, decreased sarcomere integrity and increased double-stranded DNA damage was observed (Supplemental

FIGURE 5 Mitochondrial Function Increases Upon Cotreatment With Doxorubicin and Senomorphic Drugs



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Figures 3D to 3G). Assessment of mitochondrial function by measuring oxygen consumption rates showed a significant decrease of basal respiration (FC = 0.52; $P < 0.001$), adenosine triphosphate-linked respiration (FC = 0.47; $P < 0.001$), and respiratory reserve (FC = 0.59; $P < 0.001$) in the 2-hit-exposed hPSC-CMs (**Figures 4I and 4J**). No changes were observed in the expression of genes of the oxidative phosphorylation complexes (Supplemental Figure 3C), but expression of *FIS1* (FC = 1.4; $P = 0.013$), *OPA1* (FC = 1.7; $P = 0.02$), and *PPARGC1A* (FC = 1.5; $P = 0.001$) were increased compared with control subjects (Supplemental Figure 3G). Furthermore, mitochondrial volume (FC = 1.7; $P = 0.043$) and reactive oxygen species production (FC = 1.3; $P = 0.010$) increased in 2-hit hPSC-CMs (Supplemental Figures 3H and 3I).

THE SENOMORPHIC DRUGS RESVERATROL AND AICAR IMPROVE VIABILITY AND MITOCHONDRIAL FUNCTION IN DOXORUBICIN-INDUCED CARDIOTOXICITY. Several compounds have been identified that can prevent or modulate senescence; these are known as senomorphic drugs.[18] Three senomorphic drugs were selected from the literature: resveratrol, AICAR, and rapamycin. Their efficacy was tested on the basis of their ability to improve viability of the 2-hit hPSC-CMs. Several doses were used to determine the most efficient one (Supplemental Figures 4A to 4D). Resveratrol and AICAR showed dose-dependent improvements in viability compared with the 2-hit hPSC-CMs. Resveratrol showed an optimal increase of 77% ($P = 0.012$) at 10 μmol/L, while AICAR showed a 39% increase ($P = 0.007$) at 500 μmol/L (Supplemental Figures 4B and 4C). Rapamycin was not administered during the whole treatment period, as prolonged exposure to this drug has been shown to cause toxicity.[19] Cotreatment of 2-hit hPSC-CMs with rapamycin did not result in improved viability (Supplemental Figure 4C). Furthermore, rapamycin had a detrimental effect on mitochondrial function (Supplemental Figures 4E and 4F) and was therefore excluded from further experiments.

Analysis of the Mito Stress test for AICAR and resveratrol combined with doxorubicin showed improvements. The area under the curve of the trace graphs (**Figures 5A to 5C**) showed a significant decrease (FC = 0.53; $P = 0.008$) in 2-hit-treated iPSC-CMs. Both 2-hit + resveratrol and 2-hit + AICAR did not show a significant difference from control. Further analysis showed that resveratrol rescued basal respiration and respiratory reserve. AICAR was able to rescue only respiratory reserve (**Figures 5D to 5F**).

RESVERATROL AND AICAR PREVENT SENESCENCE IN DYN-EHTs TREATED WITH DOXORUBICIN, WITHOUT IMPROVING FUNCTIONAL PARAMETERS. Using staining and western blot, we showed that in dyn-EHTs cotreated with doxorubicin and resveratrol (4-hit + resveratrol) or AICAR (4-hit + AICAR), up-regulation of p16 could be prevented (**Figures 6A to 6E**). Analysis of mRNA levels showed several differentially expressed genes. In 4-hit + AICAR, expression of mitochondrial-related genes was affected compared with 4-hit-treated tissues (**Figure 6F**). *PPARGC1A* expression was increased (FC = 122.5; $P = 0.003$), as well as *FIS1* (FC = 3.8; $P < 0.001$), *OPA1* (FC = 8.7; $P < 0.001$), *DNML1* (FC = 3.9; $P = 0.041$), *MFN2* (FC = 21.8; $P = 0.003$), and *PPARGC1B* (FC = 3.2; $P = 0.008$) (**Figure 6F**). In 4-hit + resveratrol only *PPARGC1B* expression was increased (FC = 2.4; $P = 0.017$).

Functionally, 4-hit + resveratrol did not improve diastolic length or diastolic and systolic force generation (**Figures 7A to 7C**, Videos 5 and 6). Contractile force was significantly increased compared with control subjects (56%) ($P = 0.006$) but not compared with 4-hit dyn-EHTs (Supplemental Figure 5A). Diastolic stress, systolic stress, frequency, and contractile shortening did not significantly change compared with control or 4-hit dyn-EHTs. Only contractile stress was increased over time in the 4-hit + resveratrol group ($P = 0.044$) (Supplemental Figures 5A to 5G).

Four-hit + AICAR showed increased tissue length and significant decreases in diastolic force (10%; $P = 0.004$) and systolic force (7%; $P = 0.018$) compared with control subjects, suggesting a worse functional outcome (**Figures 7D to 7F**, Videos 7 and 8). No changes were detected compared with 4-hit. Contractile force, diastolic stress, systolic stress, contractile stress, and diameter did not show changes

FIGURE 5 Continued

Mitochondrial function for doxorubicin- and senomorphic drug-treated cells was assessed using Seahorse. **(A-C)** Trace of Mito Stress tests. **(D-F)** Quantification of Mito Stress tests (basal respiration, adenosine triphosphate [ATP] production, and respiratory reserve). Linear graphs show mean ± SEM. Bar graphs show mean ± SEM and individual values. *$P < 0.05$ and **$P < 0.01$ against control. Statistical significance was determined using 1-way analysis of variance with the Bonferroni post hoc test for multiple pairwise comparisons. AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide; OCR = oxygen consumption rate; other abbreviations in **Figure 1**.

FIGURE 6 Expression of Senescence Markers Is Decreased by Cotreatment With Senomorphic Drugs

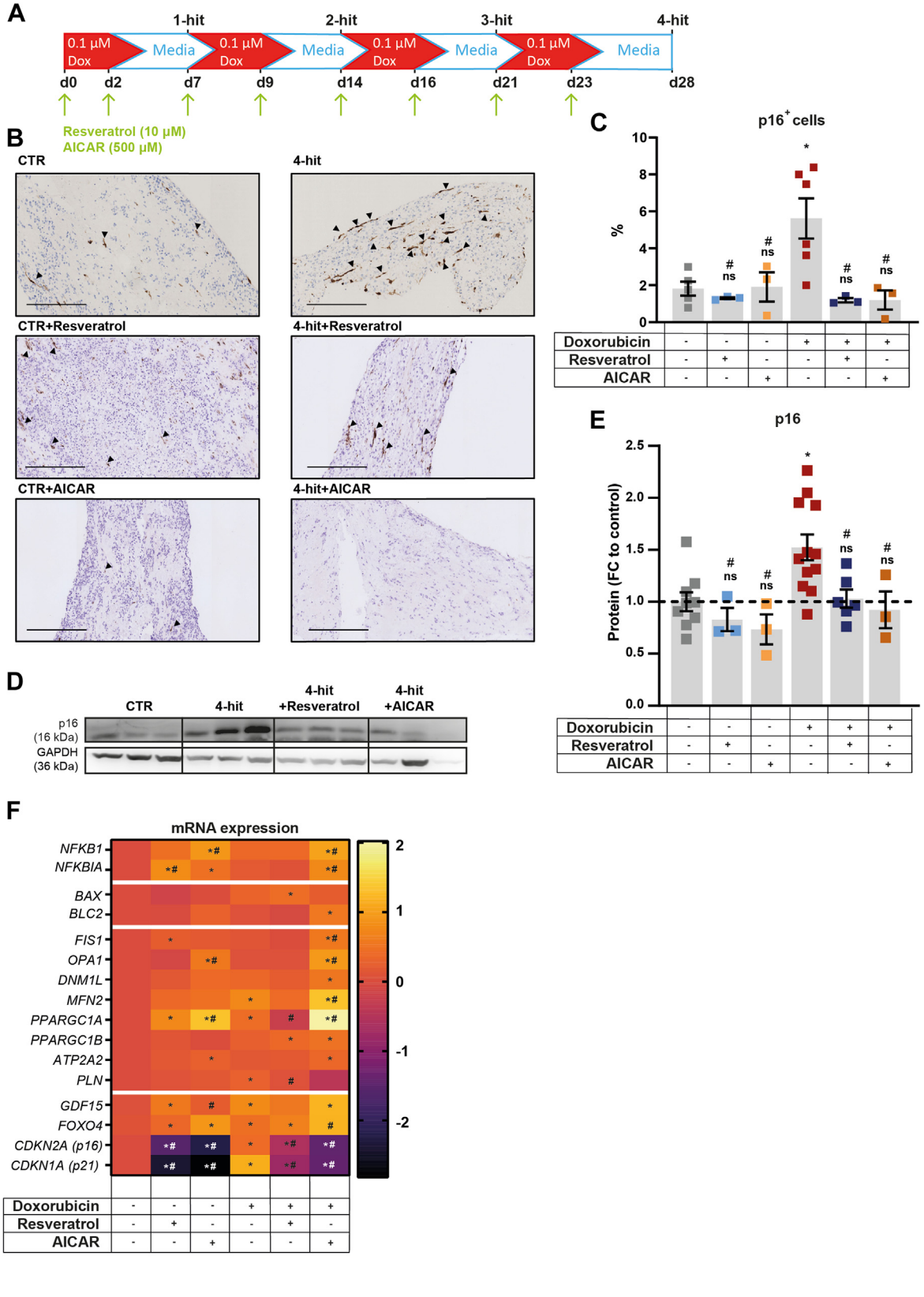

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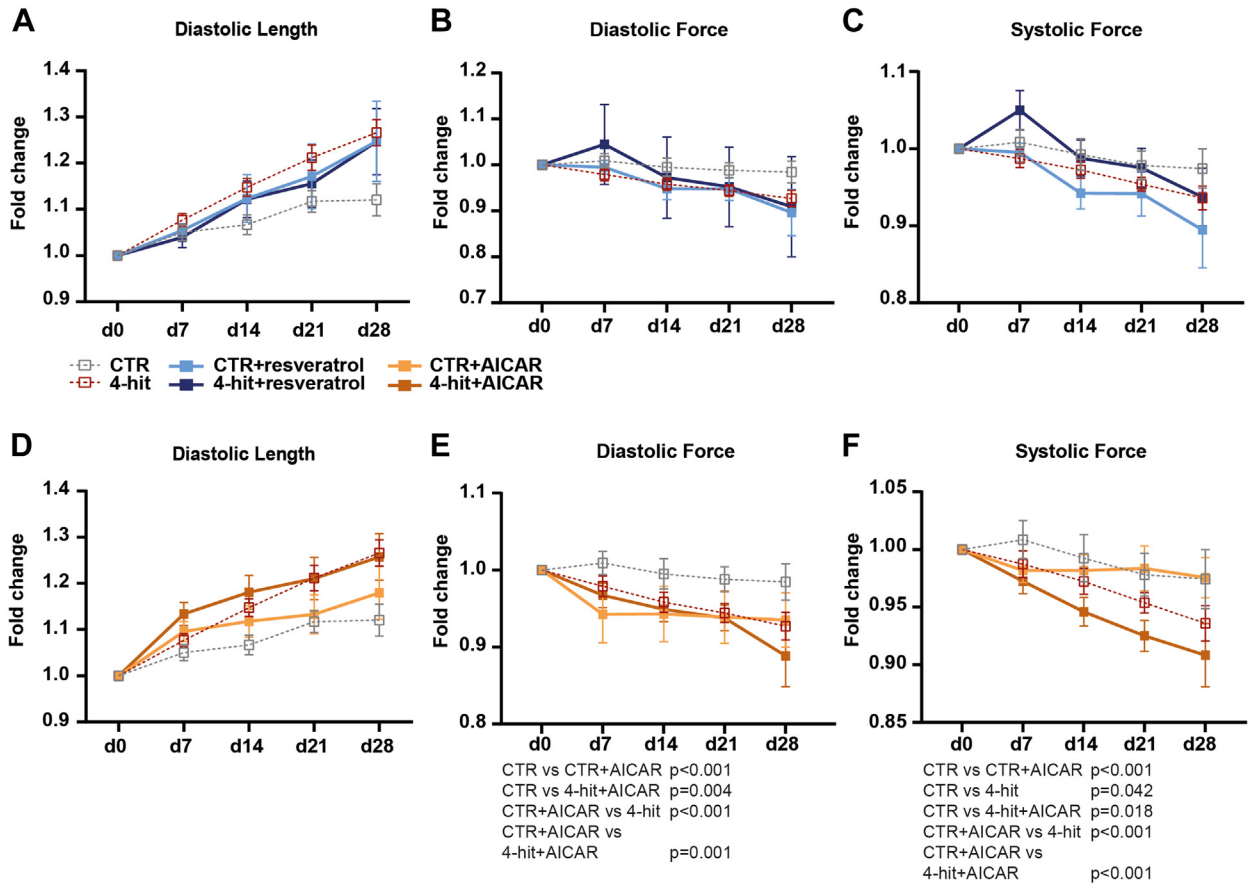
FIGURE 7 Function of 4-Hit Dyn-EHTs Cotreated With Senomorphic Drugs Is Impaired



To prevent senescence, 4-hit dyn-EHTs were cotreated with AICAR or resveratrol, and function was assessed. **(A to F)** Line graphs showing diastolic length, diastolic force, and systolic force measured in dyn-EHTs treated with doxorubicin and resveratrol **(A to C)** or AICAR **(D to F)**. Linear graphs show mean ± SEM. Significance of the functional graphs was determined using repeated-measures analysis of variance (Supplemental Table 3) and post hoc tests. Abbreviations as in **Figures 2 and 5**.

when comparing 4-hit + AICAR with control or 4-hit (**Figures 7D to 7F**). Four-hit + AICAR did show decreases over time in systolic stress ($P = 0.008$), contractile stress ($P < 0.001$), diameter ($P < 0.001$), and contractile shortening ($P < 0.001$). Furthermore, beating frequency significantly increased compared with 4-hit ($P < 0.001$) (Supplemental Figures 5H to 5N).

Masson's staining showed increased fibrosis (FC = 1.8; $P = 0.009$) (**Figures 8A and 8B**) in 4-hit + AICAR but not in 4-hit + resveratrol dyn-EHTs

FIGURE 6 Continued

Prevention of senescence by senomorphic drugs on gene expression level was assessed in 4-hit + resveratrol and 4-hit + AICAR dyn-EHTs. **(A)** Treatment scheme showing senomorphic treatments. **(B)** Representative immunohistochemical p16 staining. Scale bar: 300 μM. **(C)** Quantification of p16 stainings. **(D)** WB for p16. **(E)** Quantification of WB for p16. **(F)** mRNA expression in control and 4-hit dyn-EHTs, cotreated with resveratrol or AICAR (log-transformed FCs). Bar graphs show mean ± SEM and individual values. *$P < 0.05$ against control and $^{\#}P < 0.05$ against 4-hit. Statistical significance was determined using 1-way analysis of variance with the Bonferroni post hoc test for multiple pairwise comparisons. Abbreviations as in **Figures 1, 2, and 5**.

compared with 4-hit. mRNA expression of fibrotic markers showed an increasing trend, but this was not statistically significant (Figure 8C). Total cell count per square millimeter was also decreased for the 4-hit + AICAR group (FC = 0.51; $P = 0.021$), implying a loss of cardiomyocytes (Figure 8D). Assessment of the apoptosis markers caspase-3 and BAX/Bcl-2 ratio showed an increase in 4-hit + resveratrol dyn-EHTs (FC = 4.05 [$P < 0.0001$] and FC = 6.17 [$P = 0.004$], respectively), as well as in 4-hit + AICAR dyn-EHTs (FC = 4.32 [$P < 0.0001$] and FC = 4.88 [$P = 0.047$], respectively) (Figure 8E).

DISCUSSION

Doxorubicin remains one of the most effective chemotherapeutic drugs, its usefulness hampered by its induction of cardiotoxicity. Recent studies suggest that senescence plays a role in this side effect (Central Illustration).

DYN-EHTs TREATED WITH REPEATED LOW DOSES OF DOXORUBICIN RECAPITULATE THE PHENOTYPE IN THE PATIENT HEART. Several proteins involved in the senescence phenotype were up-regulated in patient tissue and 4-hit dyn-EHTs, including p16, p53, and phosphorylated p38MAPK. We show a 14-fold increase of $p16^+$ cells in the human samples compared with control hearts. Although highly significant, whether in patients this is causally related to cardiac dysfunction needs further study. Activation of apoptotic processes was absent in the tested conditions. Additionally, 4-hit-treated dyn-EHTs showed decreased contractile function, tissue dilatation, and increased troponin release, which are key features of doxorubicin-induced cardiotoxicity.[1] Furthermore, in 2-hit hPSC-CMs, we found an increase in DNA damage, a decline in mitochondrial respiration, and decreased sarcomeric integrity. Cotreatment with doxorubicin and resveratrol or AICAR partially increased mitochondrial respiration and prevented p16 up-regulation. However, it did not improve function in cardiac constructs and instead led to an increase in fibrosis in the 4-hit + AICAR dyn-EHTs.

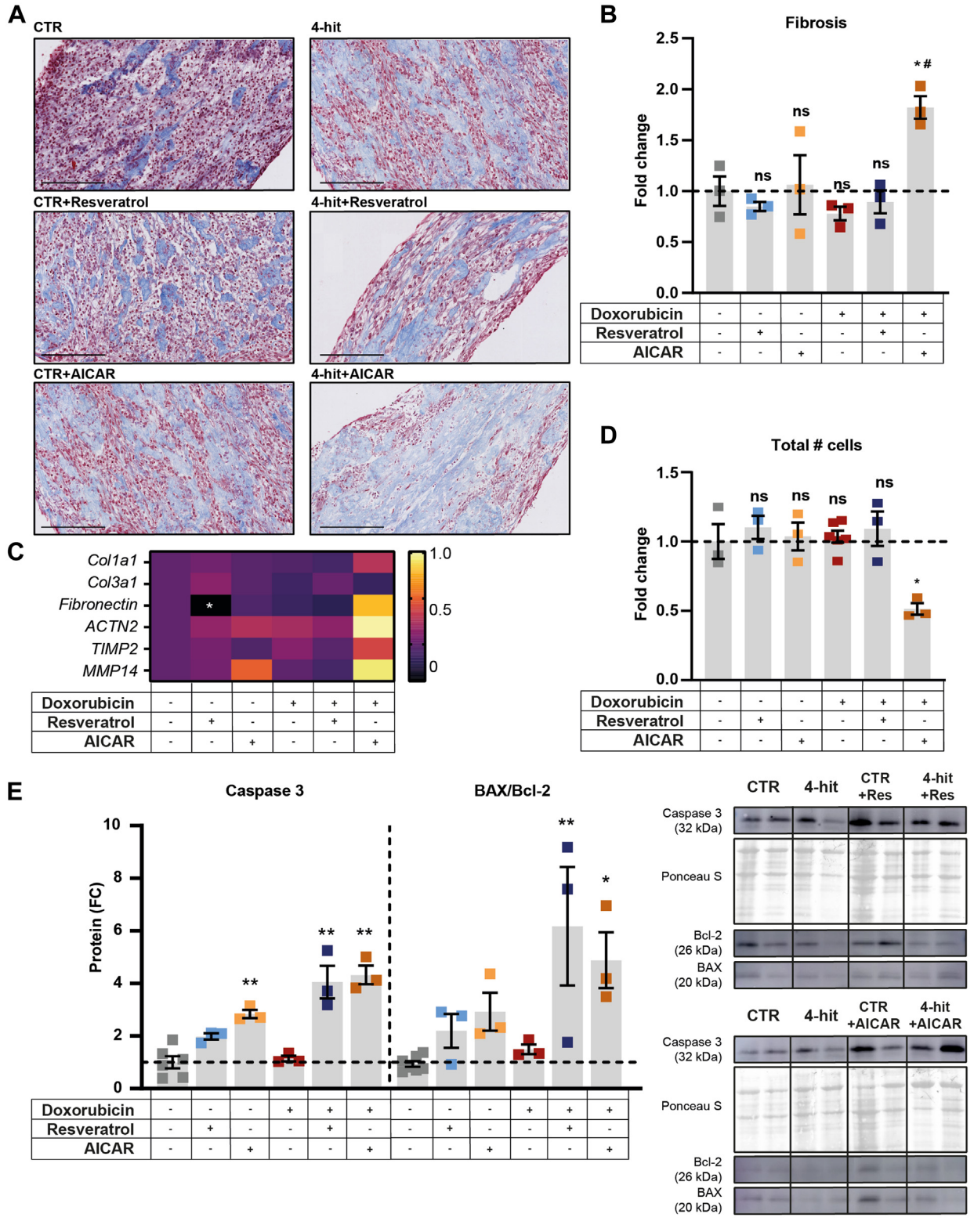
DOXORUBICIN CONCENTRATION IN IN VITRO CULTURES IS ESSENTIAL FOR OUTCOME. Animal studies often use 1 high dose (10-30 mg/kg [0.7-2.1 μmol/L][6]), while patients are exposed to multiple low doses (0.250-0.025 μmol/L[2-4]). Serum concentrations after infusion in patient serum were assessed multiple times.[2-4] Doxorubicin dose determines outcome in cardiomyocytes,[7] with higher doses resulting in apoptosis,[20] while lower doses cause senescence.[7,8] This stresses the need for models as the one proposed in this paper to correctly mimic the patient phenotype.

THE EFFECT OF SENESCENCE ON HEART FUNCTION. The effect of senescence on heart function remains controversial. Senescence prevents the development of post-myocardial infarction fibrosis and is involved in tissue repair,[21] while senescence in cardiomyocytes has been associated with functional declines.[10,22] Animal studies suggest that senomorphic drugs, such as resveratrol, AICAR, metformin, PJ34, and rapamycin, as well as senolytic drugs combined with doxorubicin treatment can rescue heart function.[11-13,23-25] So far, human data on the use of these drugs are scarce and often show neutral outcomes.[11,26-28]

In dyn-EHTs, function decreased with doxorubicin treatment, and cotreatment with resveratrol or AICAR did lead to improvement. Metformin and navitoclax were tested as well (data not shown). Metformin showed no improvements in the 2-dimensional model, while navitoclax resulted in functional decline in the dyn-EHTs. Specifically, dyn-EHTs cotreated with 4-hit + AICAR showed a decline in function combined with increased fibrosis, apoptosis, and cell loss. We speculate that cardiomyocytes exposed to doxorubicin alone become senescent—irreversible cell growth arrest and resistance to apoptosis—as a mechanism to avoid cell death and maintain the integrity of the dyn-EHT.[29] Cardiomyocyte senescence may lead to a severely dysfunctional, yet partially contractile cardiomyocyte. In the presence of a senomorphic agent, such as AICAR, cardiomyocyte senescence as a compensatory mechanism is suppressed, and cells will undergo apoptosis, which will then promote replacement fibrosis.[29] Ultimately, this will lead to decreased function. Further studies are needed to confirm the hypothesis that cardiomyocyte cell senescence plays a role in preserving cardiac function during receipt of doxorubicin.

The beneficial effects of senomorphic drugs and senolytic drugs in animal studies could be due to

FIGURE 8 **Four-Hit Dyn-EHTs Cotreated With Senomorphic Drugs Show Increased Apoptosis and Fibrosis**



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differences in systemic, in vivo responses to treatment. Additionally, our model showed differences among senomorphic drugs. Four-hit + resveratrol showed no functional differences compared with 4-hit, whereas 4-hit + AICAR showed a decline. Four-hit + resveratrol did not show increased fibrosis, whereas 4-hit + AICAR did. Both conditions reveal increased expression of apoptosis markers. We hypothesize that with time, resveratrol may cause a phenotype similar to AICAR. Four-hit + resveratrol demonstrated up-regulation of apoptosis genes, which causes cell death, fibrosis, and eventually functional decline. More research is needed to explore this hypothesis.

Another aspect to take into consideration is that experiments with senomorphic drugs are limited.[11] Because of the low regeneration rate of the heart, removing cardiomyocytes can render it unable to cope with other stressors, such as pregnancy and myocarditis. Therefore, senescent cardiomyocytes might be preferrable to fibrotic tissue.

SASP. We show mRNA expression of several SASP factors. In patients, no differences were found, whereas dyn-EHTs showed up-regulation of IL-8 and GDF-15, and fibroblasts showed up-regulation of MMP1, Edn1, IL-1b, and GDF-15. Heterogeneity is to be expected, as exposure to doxorubicin was different for the samples, and SASP expression varies with different triggers.[10] An explanation as to why so few changes were observed is that no specific SASP profile for doxorubicin-induced senescence has been established. This could be divergent from classical SASP profiles, and more research is needed.

STUDY LIMITATIONS. A limitation of this study is that our model might be too severe. Culturing cells with 21% oxygen causes them to become more senescent,[30] explaining the larger number of senescent cells in dyn-EHTs compared with the human heart samples. With fewer senescent cells in the tissue, treatment with senomorphic drugs might lead to a more beneficial outcome. However, the long-term effects on the human heart remain unexplored.

Furthermore, we cannot confirm whether doxorubicin causes senescence in patients or whether this is the result of the heart failure. Investigating this is difficult, as it requires tissue from patients who received doxorubicin but did not develop heart failure.

No fibrosis staining was carried out on the patient samples. Therefore, we cannot relate the observed lack of fibrosis in the 4-hit dyn-EHTs and the increase in the 4-hit + AICAR condition to the patient hearts.

The model has limitations as well. It is not a high-throughput model, making data generation time consuming. The proportions of fibroblasts and cardiomyocytes are not representative of the human heart. Cardiac fibroblasts make up 40% to 60% of the cell population in the heart,[31] whereas here we used 10% fibroblasts. This was done because the model does not function properly with more fibroblasts.[15]

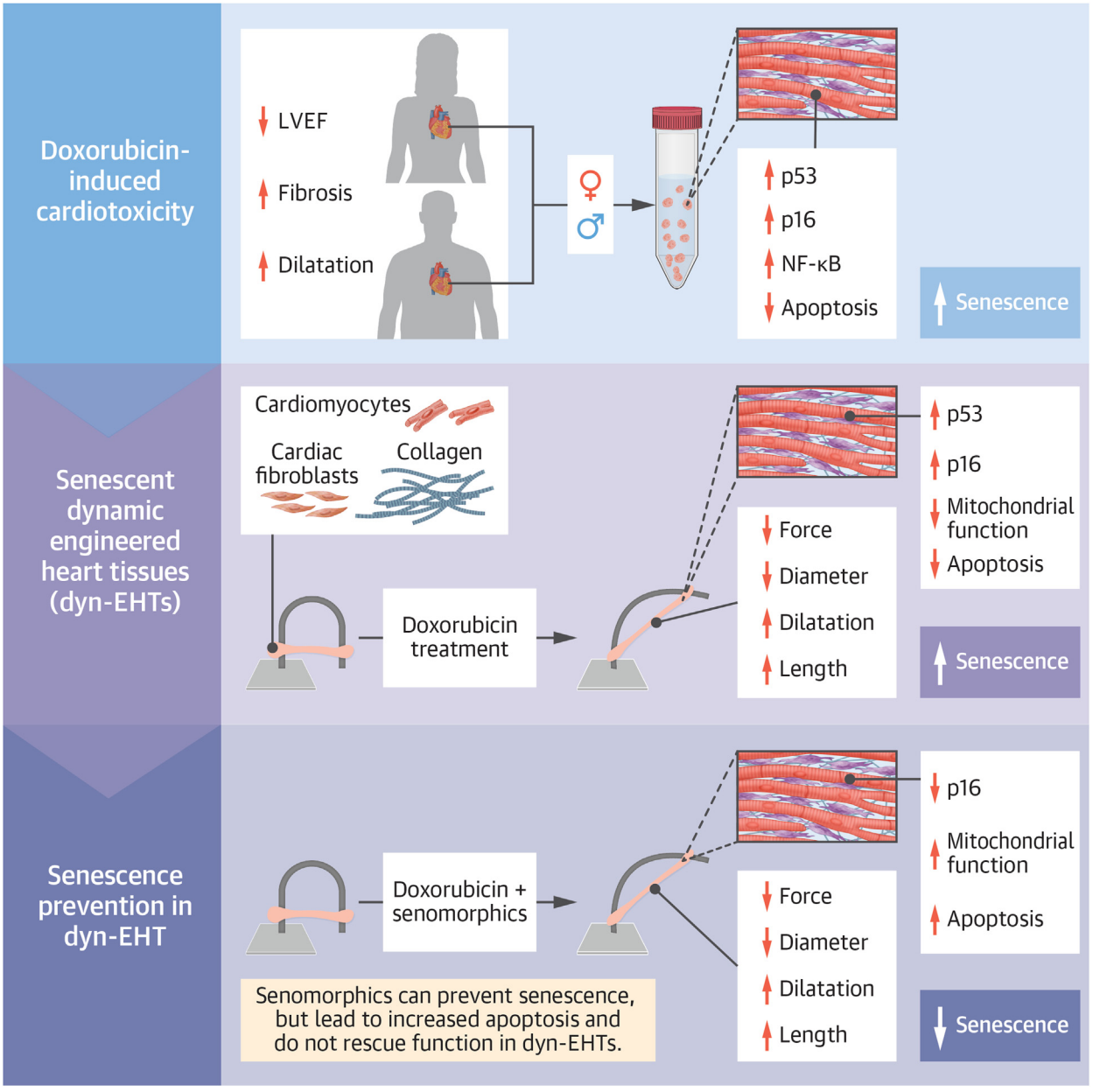
CONCLUSIONS

We established the presence of senescence in the hearts of patients with doxorubicin-induced heart failure. Additionally, we established a human dyn-EHT platform exposed to 4 clinically relevant doses (0.1 μmol/L) over the course of 4 weeks. It closely simulates key features of doxorubicin-induced heart failure, as seen in patients. AICAR and resveratrol could eliminate senescent cells and improve mitochondrial cardiomyocyte performance, but in the dyn-EHTs, they could neither prevent tissue dilatation nor improve contractile force. Cotreatment may even lead to more fibrosis. These findings suggest that prevention of senescence during doxorubicin treatment might not have the desired effect of preventing cardiotoxicity. It stresses the need for more studies regarding the mechanism, safety, and efficacy

FIGURE 8 Continued

To explain functional decline, apoptosis and fibrosis markers were assessed in cotreated dyn-EHTs. **(A)** Masson's staining, representative pictures. **(B)** Quantification of Masson's staining. Scale bar: 200 μM. **(C)** mRNA expression of fibrosis markers in control and 4-hit dyn-EHTs, cotreated with resveratrol (Res) or AICAR (log-transformed FCs). **(D)** Total number of cells per tissue area. **(E)** WB analysis of protein expression of caspase-3, BAX, and Bcl-2. Bar graphs show mean ± SEM and individual values. Significant *P* values are shown in the figure. Significance in fibrosis were determined using 1-way analysis of variance. $^{*}P < 0.05$ against control and $^{\#}P < 0.05$ against 4-hit. Abbreviations as in Figures 1, 2, and 5.

CENTRAL ILLUSTRATION **Senescence Is Involved in Doxorubicin-Induced Cardiotoxicity, and Senomorphic Drugs Did Not Improve Function**



Linders AN, et al. J Am Coll Cardiol CardioOnc. 2023;5(3):298–315.

Senescence has been suggested to be involved in doxorubicin-induced cardiotoxicity. Here, we found senescence to be present in patient tissue. A similar phenotype was observed when dynamic engineered heart tissues (dyn-EHTs) were exposed to multiple low doses of doxorubicin, separated by recovery periods. We cotreated dyn-EHTs with senomorphic drugs to prevent the phenotype, which did not improve function. LVEF = left ventricular ejection fraction.

of these drugs in patients with doxorubicin-induced cardiotoxicity.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Doxorubicin causes senescence in the human heart as well as in dyn-EHTs. Administration of senomorphic drugs during doxorubicin treatment did not improve function and in certain experiments even resulted in more fibrosis, suggesting that cotreating patients with these drugs could result in worse outcomes.

TRANSLATIONAL OUTLOOK: Further studies are needed to determine whether different treatment regimens, such as timing of administration of senomorphic drugs or senolytic drugs after doxorubicin treatment, may be a treatment strategy to prevent cardiotoxicity.

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KEY WORDS **cardiotoxicity, doxorubicin, hPSC-derived cardiomyocytes, senescence, senomorphic drugs**

APPENDIX **For supplemental tables, figures, and methods, please see the online version of this paper.**