Increasing short-term cardiomyocyte progenitor cell (CMPC) survival by necrostatin-1 did not further preserve cardiac function

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Aims
One of the main limitations for an effective cell therapy for the heart is the poor cell engraftment after implantation, which is partly due to a large percentage of cell death in the hostile myocardium. In the present study, we investigated the utilization of necrostatin-1 (Nec-1) as a possible attenuator of cell death in cardiomyocyte progenitor cells (CMPCs).

Methods and results
In a mouse model of myocardial infarction, survival of CMPCs 3 days after intra-myocardial injection was 39 ± 9% higher in cells pretreated with the Nec-1 compound. However, the increase in cell number was not sustained over 28 days, and did not translate into improved cardiac function (ejection fraction %, 20.6 ± 2.1 vs. 21.4 ± 2.5 for vehicle and Nec-1-treated CMPC, respectively). Nonetheless, Nec-1 rescued CMPCs remained functionally competent.

Conclusion
A pharmacological pretreatment approach to solely enhance cell survival on the short term does not seem to be effective strategy to improve cardiac cell therapy with CMPCs.

Keywords
Cardiac progenitor cells • Cardiac cell therapy • Cell survival • Necrostatin • BLI

1. Introduction
Cardiac cell therapy is emerging as a treatment for ischaemic heart disease. Various cell types have been successfully employed in the recovery of cardiac damage by reducing inflammation, activating endogenous regenerative responses, and, in some cases, directly participating in vasculogenesis and cardiomyogenesis.1,2 Nevertheless, many studies observed only a small percentage of engrafted cells in the myocardium upon injection, thereby possibly limiting their therapeutic effects.3–5

In the last decade, several landmark papers have disproved the notion of the human heart as a post-mitotic organ.6,7 This paradigm shift has led to the discovery of cardiac progenitor cells (CPCs), which can, to a certain extent, repopulate the heart after injury. On the basis of cell surface markers (c-kit and Sca-1),8,9 but also transcription factors (Islet-1),10 ability to efflux hoechst (side population),11 and culture conditions (cardiospheres),12 several different CPC populations have been isolated. Most of these cells are clonogenic and have the capability to differentiate into different cardiac cells such as myocytes, smooth muscle cells, and endothelial cells.13 Previously, we reported the isolation of human Sca-1+–like cardiomyocyte progenitor cells (CMPCs)14 and their potential to differentiate into cardiomyocytes and vascular structures in vitro15 and in vivo.3 Upon injection in a mouse model of myocardial infarction (MI), they preserved cardiac function. Although very promising, their long-term engraftment and subsequent direct participation in cardiac tissue regeneration was limited to ~3% of injected cells at 3 months post-injection.3 Probably, due to the hostile environment of the ischaemic myocardium, a large percentage of cells die within the first 48 h post-injection.16 Therefore, it could be of great benefit to provide a pro-survival stimulus prior to implantation.
We have shown that, under oxidative stress conditions, necrosis is the main cause of cell death in CMPCs. Although previously thought to be non-regulated, inhibiting the receptor interacting protein 1 (RIP1), a death domain protein required for activation of necrosis, cell death could be attenuated. In the current study, we attempted to improve the survival of CMPCs by the pretreatment with RIP1 inhibitor, necrostatin-1 (Nec-1), prior to injection into the myocardium and monitored cardiac function to see the effect of improved survival on myocardial damage.

2. Methods

2.1 CMPC isolation and transduction
CMPCs were isolated and propagated as previously described. For the use of human foetal tissue, individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. CMPCs were transduced with a lenti-viral construct, containing pLV-CMV-luc-green fluorescent protein (GFP), with the addition of 8 μg/mL of polybrene (Millipore). After 24 h, medium was refreshed with standard CMPC culture medium and the transduction efficiency was determined and enriched by fluorescence-activated cell sorting (FACS) sorting for GFP (BD Bioscience).

2.2 In vitro apoptosis and necrosis induction and assessment
CMPCs were pretreated with vehicle or 30 μM Nec-1 (Santa Cruz) for 30 min prior to the addition of 75 μM tert-Butyl hydroperoxide (Sigma) for 16–20 h. Analysis was performed as described previously.

2.3 Animals
All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

2.4 MI and cell transplantation
Male NOD-SCID mice, aged 10–12 weeks, were anaesthetised (ip injection; fentanyl 0.05 mg/kg; dormicium 5 mg/kg; dormitor 0.5 mg/kg) and MI was induced by ligation of the left coronary artery, as described previously. Thirty minutes after MI, 106 CMPCs, either pretreated with vehicle or Nec-1 for 30 min prior to the addition of 75 μM tert-Butyl hydroperoxide in serum-free M199 medium for 16–20 h. All cells were collected, counted, and the same number of living cells as determined by trypan blue, was used in the subsequent functional assays. Proliferation of surviving cells was monitored by WST-1 reagent (Roche), release of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) was monitored by ELISA (R&D Systems), and tubule formation was performed in micro-slide for angiogenesis. Analysis was performed on mice heart tissue harvested 1 month post-MI for each mouse. End-diastolic volume (EDV), end-systolic volume (ESV), and ejection fraction (EF) were measured by high-resolution magnetic resonance imaging (MRI; 9.4 T, Bruker Biospin) as described previously. Analysis was performed using Q-mass for mice digital imaging software (Medis) by a blinded investigator.

2.6 Magnetic resonance imaging measurements
In addition to BLI, cardiac parameters were determined prior to MI, and at 7- and 28-day post-MI for each mouse. End-diastolic volume (EDV), end-systolic volume (ESV), and fraction of EF were measured by high-resolution magnetic resonance imaging (MRI; 9.4 T, Bruker Biospin) as described previously. Analysis was performed using Q-mass for mice digital imaging software (Medis) by a blinded investigator.

2.7 Immunofluorescence
Mice were sacrificed 1 month post-MI by cervical dislocation; hearts were flushed with PBS and fixed as described previously and thereafter cut into 7 μm cryosections. All sections were numbered to identify the position of human grafts. Sections were stained as previously described and Alexa488-labelled (Invitrogen) secondary antibodies. For quantification, all human integrin-positive cells throughout each heart were scored by a blinded investigator. To calculate the area occupied by human cells, microscope images were transformed into binary image file by Image-J, and thereafter quantified.

2.8 CMPC functionality
CMPCs were pretreated with vehicle or Nec-1 for 30 min prior to the addition of 75 μM tert-Butyl hydroperoxide in serum-free M199 medium for 16–20 h. All cells were collected, counted, and the same number of living cells as determined by trypan blue, was used in the subsequent functional assays. Proliferation of surviving cells was monitored by WST-1 reagent (Roche), release of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) was monitored by ELISA (R&D Systems), and tubule formation was performed in micro-slide for angiogenesis (ibidi) coated with ECMatrix (Millipore). To induce differentiation, cells were treated with 5 μM S-azacytidine (Sigma) for 72 h in differentiation medium, followed by TGF-β1 stimulation (1 ng/mL Sigma).

2.9 Statistics
Data are presented as mean ± SEM and were compared using the two-tailed paired Student’s t-test. A difference of P < 0.05 was considered to be statistically significant.

3. Results

3.1 Nec-1 pretreatment of CMPC in vitro
Under oxidative stress conditions, CMPC mainly displayed a necrotic phenotype (Figure 1A) and by pretreatment with Nec-1, we observed a 37 ± 8% reduction in necrotic cell death in CMPCs compared with vehicle (Figure 1B). As was established previously, we did not find differences in apoptotic-mediated cell death between the two groups. Therefore, Nec-1 increased the survival of CMPCs by inhibiting necrotic cell death (Figure 1B).

3.2 Lenti-viral transduction and Nec-1 effects on CMPC functionality
Following lenti-viral transduction, GFP-positive CMPCs were enriched by FACs to attain a 92% GFP-positive cell population (see Supplementary material online, Figure S1A). Morphologically, CMPCs retained their original phenotypic appearance (see Supplementary material online, Figure S1B). Proliferation was monitored over a 16-day period, in which no changes were observed between non-transduced, transduced, and GFP-sorted CMPCs (see Supplementary
material online, Figure S1C). All cells had a doubling time of \( \approx 24 \text{ h} \), which was sustained throughout the period of the experiment.

To exclude a direct effect of Nec-1 on luciferase expression, CMPCs were pretreated either with vehicle or Nec-1 and subsequently measured by BLI. The BLI signal observed in the cells was equally strong in both groups (see Supplementary material online, Figure S2A; \( 2.0 \pm 0.4 \times 10^7 \) vs. \( 2.1 \pm 0.3 \times 10^7 \text{ ph/s/cm}^2/\text{sr} \), respectively). Furthermore, pretreated CMPCs were also passed through multiple functional assays, which tested their potency as well as the potential therapeutical mode of actions, in order to exclude off-target effects of Nec-1. Proliferation remained robust between the groups (doubling time \( \approx 24 \text{ h} \)), while tubule formation, cardiogenic differentiation potential, and the secretion of VEGF, IGF-I, and HGF remained unaffected by the Nec-1 treatment (see Supplementary material online, Figure S2B–E). Lastly, we quantified CMPC (Nec-1 and vehicle pretreated) numbers in the myocardium directly (15 min) after injection, in order to exclude a possible influence of Nec-1 on initial cell retention. The histological analysis revealed that Nec-1 did not interfere with this process. We, therefore, concluded that the viral transduction, as well as the Nec-1 pretreatment, did not affect CMPC functionality in vitro and initial cell engraftment in vivo.

### 3.3 Short-term CMPC survival in vivo

Initial experiments were carried out to test whether BLI technology could be used as a reliable measure for different CMPC numbers. In vitro, we plated CMPCs in increasingly higher numbers, which correlated strongly (\( R^2 = 0.971 \)) with the subsequent BLI measurements (see Supplementary material online, Figure S3A). Additionally, we tested this relationship in vivo by titrating different numbers of myocardial injected CMPCs, ranging from \( 2 \times 15 000 \) to \( 2 \times 240 000 \). By increasing CMPCs numbers, we observed higher BLI signals, which led to a high correlation (\( R^2 = 0.967 \)) between the two parameters (see Supplementary material online, Figure S3B). The results of these correlation experiments validated the use of BLI to monitor CMPC numbers in vivo.

To check the effect of Nec-1 on CMPC survival, mice underwent left anterior descending artery ligation and received an intramyocardial injection with (i) vehicle pretreated CMPCs; (ii) Nec-1 pretreated CMPCs. In vivo bioluminescence imaging (Figure 2A), 3-day post-injection, demonstrated a 39% higher signal (71 665 \pm 11 165 vs. 117 138 \pm 18 567 ph/s/cm\(^2\)/sr, respectively) in mice, which received the Nec-1 pretreated cells (Figure 2B). The presence of cells was confirmed by histological analysis, which revealed an increased number of human-specific cells in the myocardium of mice, which received the Nec-1 pretreated CMPCs (see Supplementary material online, Figure S3C). These data clearly showed the ability of Nec-1 to increase the short-term engraftment of CMPCs.

### 3.4 LV function and wall thickness

Cardiac functional measurements were carried out by MRI prior to MI, and at Day 7 and 28 post-MI. Baseline measurements revealed comparable ESV, EDV, and EF between the two groups (Figure 3A–D). Also, on Day 7, no significant difference could be observed in all cardiac parameters between the groups. Interestingly, in our final follow-up time of 28 days, we did not see improved cardiac recovery in the Nec-1-treated group, in which more cells initially survived. Although a small decrease...
Figure 2 Monitoring cell survival by BLI at 3 days post-injection. (A) Representative BLI image of mice, which received vehicle (nec−) or Nec-1 pretreated (nec+) CMPCs after LAD ligation. (B) Quantitative analysis of BLI signal demonstrated that Nec-1 pretreated cells had better engraftment 3 days post-injection. *P < 0.05.

Figure 3 Assessment of cardiac function by MRI at baseline, and 7 and 28 days post-injection. EDV (A), ESV (B), and per cent EF (EF %) (C) remained unaltered between vehicle and Nec-1 pretreated CMPCs at all time points. (D) Quantifications of the change in the three parameters between Day 28 and Day 7 showed no significant difference between the vehicle (nec−) and Nec-1 pretreated (nec+) CMPCs.
in EDV and ESV and an increase in EF in the Nec-1-treated group were observed, these changes were non-significant. Nevertheless, when comparing these values with those of previous injection control groups carried out in our lab, it is clear that transplantation of cells in both groups had a positive effect on the preservation of cardiac dimensions.

Preservation of the cardiac structure also plays a crucial role in preventing further remodelling. However, the thickness of the LV wall remained unaffected by increased CMPC survival (Figure 4B). Lastly, to see if there was a correlation between the observed CMPC survival by BLI and the preservation of cardiac dimensions by MRI, we plotted the EDV, or EF values from Day 28 against the BLI signal from Day 3, but also here no significant correlation could be observed (see Supplementary material online, Figure S4). Therefore, we concluded that increased short-term CMPCs survival did not translate into better preservation of cardiac dimensions.

### 3.5 Long-term CMPC survival in vivo

Engrafted CMPCs were identified in histological sections with a human-specific antibody recognizing integrin-β1, which is expressed on the human cell membrane. The transplanted cells were found back in patches located in the borderzone, as well as in the infarcted region, as described before. We identified 2.1 ± 0.5% of the initially injected vehicle pretreated CMPCs, compared with a similar 2.2 ± 0.6% of the Nec-1-treated cells (Figure 5A). Additionally to cell number, we also analysed the area occupied by the staining. However, in this quantitative analysis, we did not observe differences between the two groups at 28 days (Figure 5B).

Furthermore, for the quantification of human DNA present in the murine myocardium, we used a primer specific for human Alu sequences as previously described. We detected human DNA in all mouse hearts, however, similar levels of expression were again observed between the groups (Figure 5B). These data indicated that the observed higher short-term CMPCs engraftment in the Nec-1 group was not sustained over the 28-day follow-up period.

### 3.6 Functionality of Nec-1 ‘rescued’ CMPCs

Since we did not observe additional therapeutical beneficial effects in mice with more surviving CMPCs, we explored whether the cells that are rescued by the Nec-1 compound are as functionally competent as before the oxidative stress stimulus. For this, we put the CMPCs under oxidative stress conditions, after which they were placed in several functional assays. We did not observe any decrease in function in rescued CMPCs. Their ability to proliferate (Figure 6A) was comparable with non-rescued CMPCs (doubling time ~36 h). Similarly, the secretion of several growth factors, which seem to be important for the therapeutical benefit exerted by the paracrine factors of CMPCs, remained similar compared with non-rescued cells (Figure 6B). Furthermore, as can been seen from the tubule formation images (Figure 6D), rescued CMPCs were able to form vessel-like structures with similar size and length to those of non-rescued cells and their cardiogenic differentiation potential in vitro (Figure 6C) remain unaffected.

### 4. Discussion

In this study, we demonstrated the ability of Nec-1 to attenuate necrotic cell death in CMPCs after intra-myocardial injection. At 3 days post-injection, BLI signal and histological analysis showed an increased presence of CMPCs pretreated with the compound. However, in our histological/PCR analysis at 28 days, similar numbers of cells are recovered in both groups. Moreover, the short-term increase in cell survival did not lead to an improved preservation of LV function. In our hands, we showed that CMPCs that are treated and rescued by Nec-1 seem to be still functionally competent. Therefore, we concluded that short-term increase in survival achieved with this pharmacological approach was not an effective strategy to further improve CMPC transplantation therapy.

The lack of improvement in cardiac function seen in the group with increased short-term survival could be due to several reasons. First, it is possible that we already reached a threshold dose in the non-treated CMPC group, and that the effect of cell therapy plateaus with increased cell numbers. While CMPCs do participate to a
certain degree in vasculogenesis and cardiomyogenesis in the injured myocardium, their paracrine factors are probably their main beneficial therapeutical feature. In this context, it is feasible that there is a saturation of the receptors involved in the paracrine-mediated effects. Secondly, we observed at 28 days that the increase in cell number is not sustained by the Nec-1 pretreated group. This observation could be due to the transient nature of our pro-survival stimulus and the prevalence of cell death 3 days post-injection. The levelling off of survival benefits over the 28-day follow-up period could lead to similar therapeutical effects in both groups. Lastly, Nec-1 pretreatment potentially leads to only a relatively small increase in dosage. In the present study, as well as in multiple other studies, it has been shown that about \( \sim 15\% \) of intra-myocardial injected cells remain in the heart directly after injection. In our study, we injected \( 5 \times 10^5 \) CMPCs into the myocardium, which would lead to an acute cell engraftment of about \( \sim 5 \times 10^4 \) cells. Therefore, with our pretreatment approach, we could be observing only \( 1 \sim 3 \times 10^4 \) additional cells in the Nec-1-treated group and it might require a greater increase in cell numbers to see dose-dependent effects of CMPCs.

Interestingly, in literature, there are also numerous contradictory findings about increased cell number and the benefit it exerts on the preservation of cardiac function. This is especially apparent in the clinical arena where a consensus has yet to be reached on the dose-dependency of cardiac cell therapy. In animal models, there have been various studies investigating pretreatment or genetic manipulation options to improve cell survival after intramyocardial injection. In studies by Niagara et al. and Afzal et al., they preconditioned skeletal myoblast and MSC, respectively, with a pharmacological compound, diazoxide, and noticed improvements in cardiac function when using preconditioned cells. Although diazoxide improves cell survival, it also increases the angiogenic potential of the cells, which makes it difficult to attribute the improved LV function solely to the increase in cell number. In the study by Kutschka et al., they used a Bcl-2 viral vector to attenuate cell death in cardiomyoblasts. Although measurements by BLI showed significantly increased signals in cells overexpressing Bcl-2, no functional differences were observed between groups at the 1-month follow-up. Interestingly, a similar approach was taken by Li et al., in which a two-fold increase in survival in Bcl-2 overexpressing MSCs was associated with smaller infarct sizes and better functional recovery. Pro-survival stimuli have been investigated for CPCs as well. Hu et al. made use of a novel microRNA survival cocktail to improve the viability of CPCs, which led to a sustained higher BLI signal for up to 28 days. Although the pro-survival signalling led to a significant improvement in LV function compared with the PBS control group, they did not observe significant differences in function between the CPC-control and CPC-microRNA-treated cells. More recently, however, in a study by Liu et al., they stratified the CPC-injected mice into high and low engraftment groups based on PET imaging data. By using this early cell engraftment data from Day 1, they were able to predict the subsequent functional improvements in mice with higher cell engraftment. In regard to the 39% higher BLI signal we noted in our present study, the stratification carried out by Liu et al. resulted in an \( \sim 30\% \) higher Day 1 PET signal for the high engraftment group, however, they initially injected a higher dose \( (1 \times 10^6 \) CPCs), thereby possibly increasing the initial engraftment.
The incongruence in the above-mentioned studies can be partly attributed to the imperfection in intra-myocardial delivery of cells, especially, in the small rodent studies, but also in the large variety of cells type being studied. In the case of pro-survival stimuli, there is also a chance of off-target effects on, e.g. increased paracrine secretion or proliferation, which can influence cell behaviour, and are, therefore, not always a true representation of a dose-dependent action of cells. Lastly, the differences in the injected cell numbers (and in the case of dose–response studies the range between cell doses) also makes it difficult to compare studies. Therefore, to truly understand the influence of cell survival in the myocardium and to put our study in a broader perspective, a comprehensive large-scale dose–response study will need to be carried out for each individual cell type, which at the moment is still lacking. Nonetheless, these

Figure 6 Characterization of Nec-1 rescued cells. (A) Proliferation, as determined by WST-1, was monitored over a 3-day period. The ability of rescued CMPCs to proliferate during this time remained unchanged. (B) Secretion of VEGF, HGF, and IGF-1, in medium collected from CMPCs after 16 h incubation, was determined by ELISA. Levels of all three growth factors were similar between non- and rescued CMPCs. (C) CMPC were differentiated by 5-aza and TGF-beta stimulation. mRNA expression was determined prior to differentiation (baseline) and after 3 weeks by RT–PCR for cardiac actinin (ACTC) and troponin T (cTnT). The expression of cardiac markers was not significantly different between groups. (D) Tubule formation in matrigel is represented in terms of tubule length and size. Representative images of matrigel experiment (bottom left) illustrated the functional competence of Nec-1 rescued cells.
discrepancies also highlight the lack of knowledge in the mechanism of action behind the cardio-protective effects of cell therapy.

In conclusion, even though Nec-1 pretreatment of CMPCs increased their tolerance to oxidative stress in vitro and improved cell survival in vivo, no beneficial effects were observed on preservation of cardiac dimensions through this manipulation. Since the retention of cells after the initial wash out period is low (~15%), pro-survival strategies are, therefore, targeting an already diminished cell number, which might be impeding their therapeutic potential. New approaches using extracellular matrix or synthetic polymers for the entrapment of cells could potentially target this problem,\textsuperscript{43} and attempt to reclaim the large number of cells lost to the circulation. Survival strategies could conceivably be more useful in such settings, as they can exert their effects on larger number of cells.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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