#1160

Mcl-1 Knockdown Causes a Sub-Lethal Injury in Cultured Human iPSC-Cardiomyocytes

L. Guo[1], J. Mussio[1], *M. Furniss*[1], J. Hamre[1], *S. Eldridge*[2], R. Parchment[1], and *M. Davis*[2]

[1]Laboratory of Investigative Toxicology, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD, [2]Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD

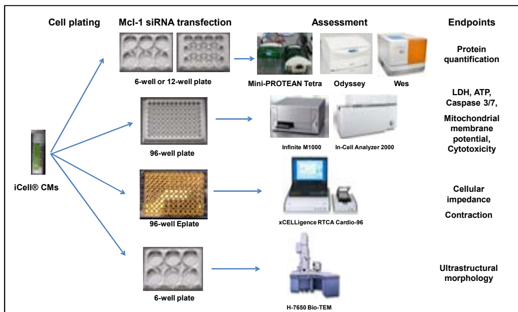
Abstract

Chemotherapeutics targeting the bcl-2 family member, Myeloid cell leukemia sequence 1 (Mcl-1) are being evaluated as potential anticancer therapies due to the role of Mcl-1 in promoting survival of Myc-induced cancer. It has become apparent that both the anti-apoptotic and mitochondrial functions of Mcl-1 play important roles in promoting the survival of cancer cells, hematopoietic stem cells, mitochondrial homeostasis and induction of autophagy in the heart. We characterized the role of Mcl-1 in maintaining contractile function and structural integrity of human iPSC-derived cardiomyocytes in vitro and examined whether the pathology observed in Mcl-1 knockout mice can be recapitulated in vitro. Using Mcl-1 siRNA, we knocked down (KD) Mcl-1 Long (Mcl-1L) protein expression by over 80%, without consistent effects on Mcl-1 Short (Mcl-1S). Mcl-1L KD was typically associated with a decrease in beat amplitude and cellular impedance by 20%, and a reduction in cellular ATP by 11%. We also observed increased LDH activity in the cellular medium of ~70% compared to non-targeted siRNA controls. There were no measurable changes in mitochondrial membrane potential (globally), but there were observable changes in mitochondrial morphology and autophagosome or mitophagosome in electron microscopy images. Interestingly, the KD of Mcl-1L was associated with an increase in activated caspase-3/7. A pan caspase inhibitor, Z-VAD, blocked activation of caspase 3/7, but had no other measurable effect on other cellular parameters. Finally, Mcl-1L KD only weakly potentiated doxorubicin-induced changes in cell impedance, ATP, beat rate and beat amplitude. Additional investigations of the role of MCL-1 in regulation of cardiac viability and function in human iPSC-derived cardiomyocytes are needed. Funded by NCI Contract # HHSN261200800001E.

Methods and Materials

Experimental Procedure

HiPSC-derived cardiomyocytes (CMs, Cellular Dynamics International) were plated on cell culture plates or 96-well E-plates (ACEA Biosciences) and maintained per manufacturer's instructions; Mcl-1 knockdown was achieved with ON-TARGETplus SMARTpool Human Mcl-1 siRNAs (GE Dharmacon) and confirmed by Western Blot with Mini-PROTEAN Tetra Cell Electrophoresis System (BIO-RAD), Odyssey® infrared image system (LI-COR Bio), or Wes™ size-based electrophoresis immunoassay Simple Western system (ProteinSimple); Cell injury, viability or contractile function were assessed using CytoTox 96® Non-Radioactive Cytotoxicity Assay Lactate Dehydrogenase (LDH) kit, CellTiter-Glo® Luminescent Cell Viability Assay (ATP) kit or Caspase-Glo® 3/7 Assay kit (Promega), and Cell Meter MeterTM JC-10 Assay kit (AAT Bioquest) with TECAN Infinite® M1000 Plate Reader (TECAN), IN Cell Analyzer 2000 (GE Healthcare), xCELLigence RTCA Cardio (ACEA) and Hitachi H-7650 Bio-TEM (Hitachi High Technologies).



Data Analysis

Measurements were averaged and presented as mean ± SEM (n ≥ 3 replicate wells or separate experiments). Student's *t* test with $P < 0.05$ was used to define statistical significant changes between groups.

Kinetics of siRNA-mediated Mcl-1 Knockdown (KD)

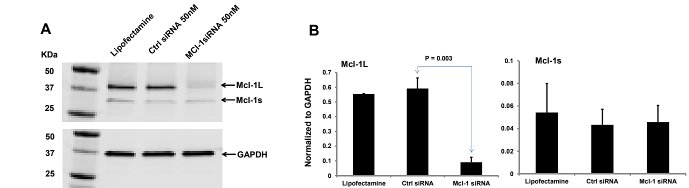


Figure 1 Quantification of siRNA-mediated Mcl-1 knockdown using Mini-PROTEAN Tetra Cell Electrophoresis System. **A.** hiPSC-CMs cultured in 6-well plates were exposed to Lipofectamine/Opti-MEM (transfection vehicle), 50 nM control or Mcl-1 targeted siRNAs for 72 hours; cell lysates were then collected with RIPA buffer, and loaded at 30 µg/lane protein; the separated proteins were then probed with Mcl-1 Ab (sc-819, diluted 1:200) and GAPDH Ab (diluted 1:2000), and resulted in two bands representing Mcl-1L and Mcl-1s at 40 and 32 KDa, respectively, and one band for GAPDH at 37 KDa. **B.** Mcl-1 expression was normalized to GAPDH and the efficiency of knockdown by Mcl-1 siRNA was calculated as 85% compared to control siRNAs (Mean ± SEM, n = 3 separate experiments).

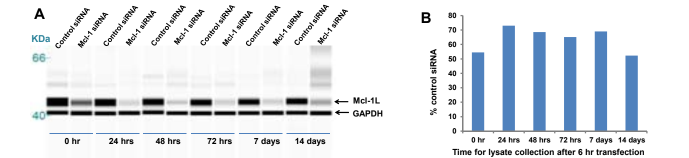


Figure 2 Quantification of siRNA-mediated Mcl-1 knockdown using the Wes™ Simple Western system. **A.** hiPSC-CMs cultured in 12-well plates were exposed to Lipofectamine/Opti-MEM (transfection vehicle), 50 nM control or Mcl-1 targeted siRNAs for 6 hours; cell lysates were then collected with RIPA buffer at 0 to 14 days post-wash, and protein analyzed; the separated proteins were probed with Mcl-1 Ab (sc-819, diluted 1:25) and GAPDH Ab (diluted 1:100), and resulted in one band at 46 KDa and another one at 41 KDa, representing Mcl-1 and GAPDH, respectively. **B.** Mcl-1 expression was normalized to GAPDH and the knockdown efficiency of Mcl-1 siRNA was calculated as % of control siRNAs.

Mcl-1 KD Increases Caspase Activity and Membrane Permeability

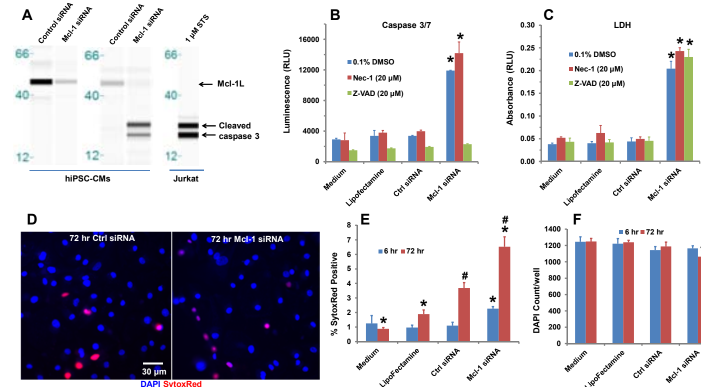


Figure 3 Detection of Mcl-1 knockdown (KD)-induced caspase 3/7 activation and increased membrane leakage. **A.** hiPSC-CMs were transfected with 50 nM siRNAs for 72 hrs, lysates were collected; protein was analyzed using the Wes™ Simple Western system and detected with Mcl-1 Ab (sc-819) and cleaved caspase-3 Ab (CS9664) at 1:25 dilution, along with lysates collected from Jurkat cells treated with 1 µM staurosporine (STS) for 6 hrs. Two bands at 23 and 28 Kda represent cleaved caspase-3 I and II, respectively. **B** and **C.** Mcl-1 KD with 50 nM siRNAs for 72 hrs increased caspase-3/7 activity and LDH release that were not affected by the necroptosis inhibitor Necrostatin-1 (Nec-1, 20 µM). Increased caspase-3/7 activity, but not LDH leakage, was abolished by the pan caspase inhibitor Z-VAD-FMK (20 µM). **D**, **E** and **F.** Mcl-1 knockdown induced membrane leakage and cell loss were manifested by the increased nuclear stain with SYTOX Red and reduced DAPI counts. *, # p < 0.05 vs. the Ctrl siRNA or 6 hr transfection group; mean ± SEM, n = 3 or 4 wells.

Mcl-1 KD Reduces Cellular Impedance and ATP

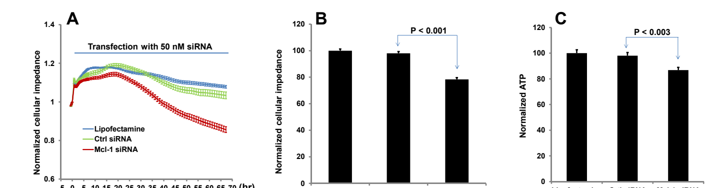


Figure 4 Effects of Mcl-1 knockdown (KD) on cellular impedance and ATP. **A.** Real-time monitoring of cellular impedance during 72 hour transfection using xCELLigence RTCA Cardio system; readings from each well were normalized to pre-transfection period. Each trace represents the mean ± SEM of 16 wells in a single eplate. **B** and **C** show the cellular impedance and intracellular ATP measured at the end of 72 hours transfection. Mcl-1 knockdown resulted in a reduction of cellular impedance and ATP by 20% and 11%, respectively. Data points for each bar represent the mean ± SEM of 39 wells from 3 replicate experiments.

Mcl-1 KD Leads to Ultrastructural Changes

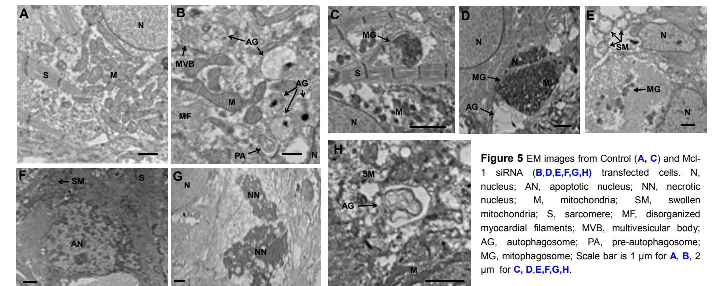


Figure 5 EM images from Control (**A**, **C**) and Mcl-1 siRNA (**B,D,E,F,G,H**) transfected cells. N, nucleus; AN, apoptotic nucleus; NN, necrotic nucleus; M, mitochondria; SM, swollen mitochondria; S, sarcomere; MF, disorganized myocardial filaments; MVB, multivesicular body; AG, autophagosome; PA, pre-autophagosome; MG, mitophagosome; Scale bar is 1 µm for **A**, **B**, 2 µm for **C**, **D**,**E**,**F**,**G**,**H**.

Mcl-1 KD Modulates Doxorubicin Toxicity

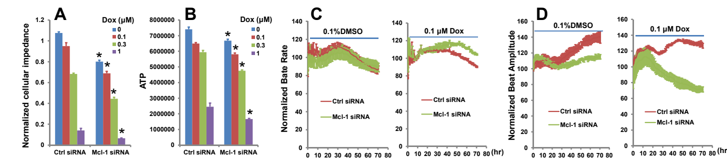


Figure 6 Effects of Mcl-1 knockdown (KD) on doxorubicin (Dox) toxicity. Cells were transfected with control (Ctrl) or Mcl-1 siRNA for 72 hours; the cellular impedance, beat rate and beat amplitude were monitored in real-time, and ATP was measured at the end of transfection. Mcl-1 knockdown weakly potentiated Dox-induced reduction in cellular impedance (**A**) and ATP (**B**), increased beat rate (**C**) and further decreased the beat amplitude (**D**) in cells exposed to Dox. N = 3 wells for each group, * P < 0.05 compared to Ctrl siRNA group.

Discussion and Conclusions

- Mcl-1 expression in hiPSC-CMs can be down-regulated effectively (> ~70%) with targeted siRNA for more than 7 days in culture.
- Mcl-1 knockdown (KD) was associated with a small increase in caspase-3 cleavage, caspase 3/7 activity, LDH release, plasma membrane permeability, spontaneous beating rate, and a small decrease in cell count, ATP content, cellular impedance or beating amplitude.
- Mcl-1 KD induced LDH release, membrane permeability, ATP reduction and cell loss were not prevented by necroptosis inhibitor Necrostatin-1 or pan-caspase inhibitor Z-VAD, even though Z-VAD effectively inhibited the increase in Mcl-1 KD induced caspase 3/7 activity.
- Mcl-1 KD modulated doxorubicin mediated cardiomyocyte injury in an additive or weak potentiation manner.
- Mcl-1 KD led to degenerative ultrastructural changes in mitochondria, nuclei, sarcomeres, and autophagic vesicles.
- Continuing efforts are needed to further characterize the role of Mcl-1 in hiPSC-CMs.

Thomas, et al. (2010) FEBS Letters 584:2981-2989
Wang, et al. (2013) Genes & Development, 27:1351-1364
Belmar & Fesik. (2015) Pharmcol Ther 145:76-84.
Thomas, et al. (2013) Genes & Development, 27: 1365-1377

Disclaimers: This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Contract HHSN261200800001E - Funded by the National Cancer Institute