Protective Effects of Cyanidin-3-Glucoside on Doxorubicin-Induced Injury in Differentiated Human AC16 Cardiomyocytes

Chad Clark Research Scholar, Lindsey Miller PhD
Lincoln Memorial University, DeBusk College of Osteopathic Medicine, Knoxville, TN 37932

Abstract

Background: Doxorubicin (Dox) is an effective anthracycline whose use is limited by dose-dependent cardiotoxicity involving oxidative stress and impaired Nrf2-regulated defenses. Cyanidin-3-glucoside (C3G), a dietary anthocyanin, activates Nrf2-linked antioxidant and anti-apoptotic pathways in several models and could reduce Dox-induced injury in human cardiomyocyte-like cells.
Objectives: (1) Differentiate AC16 cells and confirm phenotypic changes with Rt-qPCR for hexokinase 2 (HK2) and bone morphogenic protein 2 (BMP2); (2) test whether C3G pre- and/or co-treatment alters Dox-induced loss of viability; (3) identify if C3G regimens worth further cardioprotective testing.
Methods: AC16 cells were grown, differentiated for 7 days, then serum-starved before treatment. Differentiation was assessed by morphology and RT-qPCR for HK2 and BMP2 normalized to Actin Beta (ACTB) ($2^{-\Delta\Delta Cq}$). Differentiated cells were treated for 48 h with Dox (0–2.5 µM) ± C3G (5–1,000 ng/mL) as pre- and/or co-treatments; viability was measured by XTT and analyzed by one-way ANOVA with Tukey and Dunnett post hoc tests (α=0.05).
Results: Differentiation produced clear morphological changes and significantly increased HK2 (p=0.0002), while BMP2 showed a modest, qualitative increase. Dox caused a significant, dose-dependent fall in XTT absorbance, with 2–2.5 µM consistently reducing viability vs 0 µM; no C3G regimen differed significantly from its Dox-only control at 1 or 2 µM Dox.
Conclusions: Differentiated AC16 cells provide a practical human cardiomyocyte-like model for Dox cardiotoxicity. Under the conditions tested, C3G did not significantly modify Dox-induced loss of viability.

Introduction

Doxorubicin is a widely used anthracycline chemotherapeutic, with cardiotoxicity that can occur acutely or emerge years after treatment[1], making cardioprotection an important area of investigation. Oxidative stress is a major contributor to this toxicity[2], and the Nrf2 pathway regulates antioxidant[3], iron-handling, and anti-apoptotic responses[4] that may influence cardiomyocyte survival during Dox exposure.

Cyanidin-3-glucoside (C3G) is a naturally occurring anthocyanin found in blueberries[5] that has demonstrated antioxidant activity and effects on Nrf2-associated targets[6] including Bcl-2 (anti-apoptotic regulator)and GPX-4 (lipid peroxide-reducing antioxidant enzyme), with strong support for increasing Nrf2 modulated FPN1(ferroportin iron exporter) expression[9].C3G is a plausible nutraceutical candidate for reducing Dox-induced cellular injury in the heart as it has been validated in both human and non-human models.

AC16 cells are an immortalized human cardiomyocyte cell line[10], providing a practical platform that can be maintained in a proliferative state or shifted toward a more differentiated cardiomyocyte-like phenotype through media manipulation. The purpose of this project was to explore possible cardioprotective effects of C3G against Dox-induced cytotoxicity in differentiated AC16 cardiomyocytes.

Methods

AC16 cells were thawed from liquid nitrogen, plated in T-75 flasks with growth medium (DMEM/F12, 12.5% FBS, 1% PCN), and expanded to ~70% confluence in a CO_2-controlled incubator. Confluent cells were sub-cultured or plated into treatment plates, then switched to differentiation medium (DMEM/F12, 2% horse serum, 1% insulin-transferrin-selenium) for 7 days. Differentiation was assessed morphologically **(Figs. 1–3)** and by RT-qPCR using ACTB as a housekeeping gene and HK2 and BMP2 as markers **(Figs. 4–6)**.
The same markers were examined at 3 and 7 days of differentiation; 3-day samples did not show significant changes.
To test Dox and C3G effects, ~70% confluent cells were differentiated for 7 days, then switched to serum-free DMEM/F12 for 24 hours, with some wells receiving C3G pre-treatment during this period. Fresh serum-free medium containing Dox and/or C3G was then added for 48 hours, after which samples were collected and stored at −80 °C until analysis.

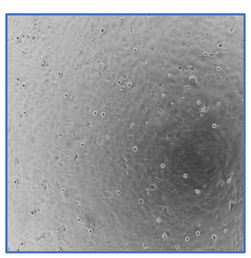

Fig.1 Confluent AC16 in Growth media

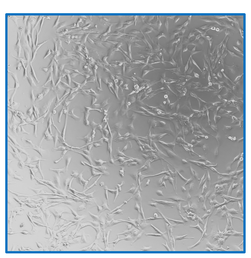

Fig. 2 AC16 cells at Differentiation Day 0

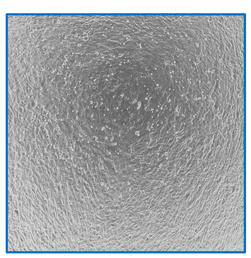

Fig. 3 AC16 cells at Differentiation Day 6

Figs 1-3:
10x magnification of plated AC16 cells. **Fig. 1** AC16 growing to confluence with growth media. **Fig. 2** is AC16 cells at ~ 60% confluence at time of switching from growth media to differentiation media. **Fig. 3** is same well as Fig. 2 at day 6 of differentiation media exposure.

Results

Dox produced a clear, dose-dependent reduction in XTT absorbance, with 2–2.5 µM causing the most consistent loss of viability compared with 0 µM **(Fig. 7)**. C3G alone (5–1,000 ng/mL) had little effect on viability. When combined with Dox, C3G pre- and co-treatments caused only small, inconsistent changes in XTT signal, and Tukey/Dunnett post hoc tests found no regimen that significantly improved or worsened viability at either 1 µM or 2 µM Dox **(Figs. 8–9)**. In parallel RT-qPCR experiments, housekeeping genes showed large Cq variability across groups, preventing reliable $2^{-\Delta\Delta Cq}$ normalization of Nrf2-related targets.

Differentiation

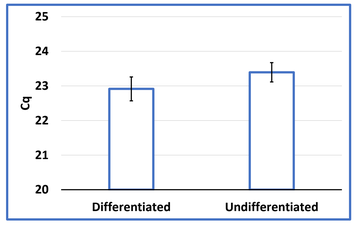

Fig.4 ACTB Rt-qPCR expression in Cq

Figs. 4-6:

RT-qPCR was used to compare undifferentiated vs 7-day differentiated AC16 cells. **Fig. 4** shows ACTB Cq values, which were similar between groups and support its use as a housekeeping gene for these comparisons. **Fig. 5** shows a significant increase in HK2 expression in differentiated cells, consistent with a more cardiomyocyte-like metabolic phenotype (p = 0.0002, $2^{-\Delta\Delta Cq}$ analysis). **Fig. 6** shows an increase in BMP2 expression, but undifferentiated samples lacked sufficient results for statistical analysis.

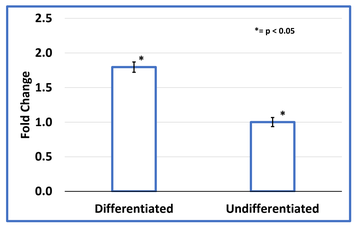

Fig.5 Fold change in HK2 Rt-qPCR expression

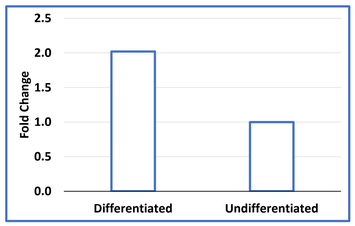

Fig.6 Fold change in BMP2 Rt-qPCR expression

Cell Survival / Viability

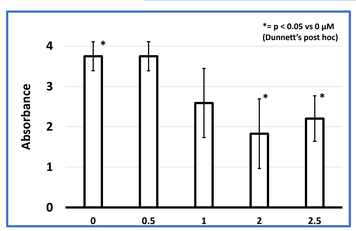

Fig.7 Cell Viability at 48 hours with increasing Dox µM

Figs. 7-9:

Fig.7 One-way ANOVA showed a significant overall effect of Dox dose, and Dunnett's test identified 2–2.5 µM Dox as significantly lower than 0 µM.

Figs. 8 & 9 In contrast, although ANOVAs for C3G regimens were significant, Tukey/Dunnett post hoc tests found no individual C3G condition that significantly differed from Dox alone.

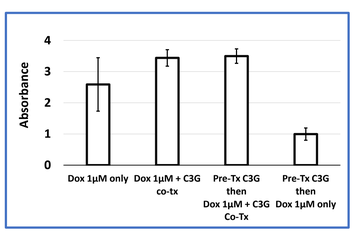

Fig 8. Cell Viability at 48 hours with Dox 1µM and C3G 1,000 ng/ml Treatments

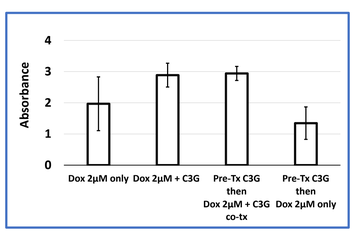

Fig 9. Cell Viability at 48 hours with Dox 2µM and C3G 1,000 ng/ml Treatments

Discussion

Discussion:
Differentiation of AC16 cells produced clear morphological changes and a significant increase in HK2 expression, supporting this system as a human cardiomyocyte-like model for Dox cardiotoxicity and cardioprotective screening. Dox caused a dose-dependent reduction in XTT absorbance, with 2–2.5 µM producing the most consistent loss of viability, while C3G pre- and co-treatments caused only small, variable shifts that were not statistically significant versus Dox alone after multiple-comparison correction. Variable housekeeping gene expression across Dox/C3G conditions prevented reliable normalization of Nrf2-related RT-qPCR targets, limiting molecular interpretation. Overall, differentiated AC16 cells appear suitable for modeling Dox-induced injury, but under the dosing and timing used here, C3G did not provide robust protection, underscoring the need for optimized regimens, larger sample sizes, and improved reference-gene strategy in future work.

Limitations:
AC16 cells, although derived from human cardiomyocytes, remain an immortalized line with incomplete maturation, and the small sample size plus unstable housekeeping expression weakens the strength of conclusions.

Future Studies:
- Test alternative C3G dosing schedules and combinations, including higher Dox doses, longer treatment, and extended pre-treatment.
- Identify more stable housekeeping genes and expand RT-qPCR panels for Nrf2 targets.
- Add complementary assays (e.g., flow cytometry, western blot, multiplex immunoassays).
- Extend work to additional cardiomyocyte models or human iPSC-derived cardiomyocytes to assess translational relevance.

References

1. Belger C, Abrahams C, Imamdin A, Lecour S. Doxorubicin-induced cardiotoxicity and risk factors. *Int J Cardiol Heart Vasc*. 2023;50:101332. Published 2023 Dec 27. doi:10.1016/j.ijcha.2023.101332
2. Chatterjee K, Zhang J, Honbo N, Karliner JS. Doxorubicin cardiomyopathy. Cardiology. 2010;115(2):155-162. doi:10.1159/000265166.
3. Li S, Wang W, Niu T, et al. Nrf2 deficiency exaggerates doxorubicin-induced cardiotoxicity and cardiac dysfunction. *Oxid Med Cell Longev*. 2014;2014:748524. doi:10.1155/2014/748524
4. Kong CY, Guo Z, Song P, et al. Underlying the Mechanisms of Doxorubicin-Induced Acute Cardiotoxicity: Oxidative Stress and Cell Death. *Int J Biol Sci*. 2022;18(2):760-770. Published 2022 Jan 1. doi:10.7150/ijbs.65258
5. Rahman, S., Mathew, S., Nair, P., Ramadan, W. S., & Vazhappilly, C. G. (2021). Health benefits of cyanidin-3-glucoside as a potent modulator of Nrf2-mediated oxidative stress. *Inflammopharmacology*, *29*(4), 907–923. https://doi.org/10.1007/s10787-021-00799-7
6. Li, X., Mu, J., Lin, Y., Zhao, J., & Meng, X. (2021). Combination of cyanidin-3-O-glucoside and cisplatin induces oxidative stress and apoptosis in HeLa cells by reducing activity of endogenous antioxidants, increasing bax/bcl-2 mRNA expression ratio, and downregulating Nrf2 expression. *Journal of food biochemistry*, *45*(7), e13806. https://doi.org/10.1111/jfbc.13806
7. Zhao L, Zhou N, Zhang H, et al. Cyanidin-3-O-glucoside and its metabolite protocatechuic acid ameliorate 2-amino-1-methyl-6-phenylimidazo4,5 — *b*pyridine (PhIP) induced cytotoxicity in HepG2 cells by regulating apoptotic and Nrf2/p62 pathways. *Food Chem Toxicol*. 2021;158:112582. doi:10.1016/j.fct.2021.112582
8. Shan X, Lv ZY, Yin MJ, Chen J, Wang J, Wu QN. The Protective Effect of Cyanidin-3-Glucoside on Myocardial Ischemia-Reperfusion Injury through Ferroptosis. *Oxid Med Cell Longev*. 2021;2021:8880141. Published 2021 Feb 6. doi:10.1155/2021/8880141
9. Harada, N., Kanayama, M., Maruyama, A., Yoshida, A., Tazumi, K., Hosoya, T., Mimura, J., Toki, T., Maher, J. M., Yamamoto, M., & Itoh, K. (2011). Nrf2 regulates ferroportin 1-mediated iron efflux and counteracts lipopolysaccharide-induced ferroportin 1 mRNA suppression in macrophages. *Archives of biochemistry and biophysics*, *508*(1), 101–109. https://doi.org/10.1016/j.abb.2011.02.001
10. Davidson, M. M., Nesti, C., Palenzuela, L., Walker, W. F., Hernandez, E., Protas, L., Hirano, M., & Isaac, N. D. (2005). Novel cell lines derived from adult human ventricular cardiomyocytes. *Journal of molecular and cellular cardiology*, *39*(1), 133–147. https://doi.org/10.1016/j.yjmcc.2005.03.003

Acknowledgements

This research project was supported by the 2025-2026 LMU-DCOM Scholar Award.

Thank you to Dr. Lindsey Miller for supplying AC16 cells, as well as mentorship and encouragement throughout this project.

Thank you to Ms. Helen Sowder and Mr. Zachary Wells for assistance with data collection and data interpretation.