

Combinatorial resveratrol and quercetin polymeric micelles mitigate doxorubicin induced cardiotoxicity *in vitro* and *in vivo*

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Chemical compounds studied in this article

Doxorubicin Hydrochloride (PubChem CID: 443939); Curcumin (PubChem CID: 969516);

Quercetin dihydrate (PubChem CID: 16212154); Resveratrol (PubChem CID: 445154)

Introduction

Doxorubicin hydrochloride, also known as Adriamycin for Injection, USP (ADR) is an effective anthracycline antibiotic used to treat lymphomas, breast, and ovarian cancers.[1] In its current formulations, it exhibits extensive cardiotoxic side effects which limits the cumulative lifetime dose to 450 to 550 mg/m², depending on patient specific factors.[1] Though the exact mechanism of cardiotoxicity is still unknown, it is believed the redox cycling of the quinolone ring in ADR generates reactive oxygen and nitrogen species resulting in extensive cardiac damage, limiting its full potential as a therapeutic option.[1, 2] Combining ADR with other agents to mitigate its highly cardiotoxic adverse effects as a chemotherapeutic strategy has strong merit.

Various natural products have demonstrated cardioprotective effects *in vitro* and *in vivo*. [3, 4] Among such natural products, resveratrol (RES) and quercetin (QUE), commonly found in grapes, have demonstrated cardioprotective effects when dosed individually with ADR.[5, 6] RES exhibits cardioprotective effects through various mechanisms, such as antioxidant and free radical scavenging, and impeding lipid peroxidation *in vitro* or *in vivo*. [5, 7] RES has also demonstrated chemopreventative, and chemotherapeutic effects *in vitro* and *in vivo*. [5, 8] In addition, a recent literature review has highlighted its role as a chemosensitizer when used in rodents in combination with conventional chemotherapeutics.[9] Thus far, the clinical outcomes of RES are limited due to its low aqueous solubility (30 µg/mL) and low oral bioavailability (<1%). [7, 10-12] QUE is a strong antioxidant and free radical scavenger which affords protection against cardiovascular disease.[4, 13] QUE has been shown to cause cell cycle arrest in cancer cell lines at the G₀/G₁ phase.[14] However, QUE has low aqueous solubility (2.2 µg/mL) and an oral bioavailability of less than 1% due to high first pass metabolism, limiting its clinical use.[15, 16]

Pluronics[®] are triblock copolymers consisting of a polypropylene oxide (PPO) chain flanked with two polyethylene oxide (PEO) chains that can spontaneously self-assemble into core/shell structures known as polymeric micelles.[17] These polymeric micelles have hydrophobic cores which help solubilize compounds with poor aqueous solubility. Currently, a Pluronic[®] based micellar delivery system for doxorubicin is in Phase III clinical trials.[18] Pluronic[®] micelles have also demonstrated longer circulating times for the encapsulated molecules *in vivo*. [19] We hypothesize that the combination of RES and QUE in Pluronic[®] micelles (mRQ) when co-administered with ADR, will be cardioprotective *in vitro* and *in vivo*, while maintaining or increasing the efficacy of ADR against cancer cell lines *in vitro*. The objective of this work is two-fold. First, evaluate the cardioprotective effect of mRQ co-administered with ADR in *in vitro* and *in vivo* models. Secondly, assess the anti-proliferative effect of the mRQ and ADR combination *in vitro* in ovarian cancer cells.

Materials

QUE was purchased from Alfa Aesar (Ward Hill, MA). RES and ADR were obtained from TCI (Portland, OR) and LC Labs (Woburn, MA), respectively. Lutrol F-127 Pluronic[®] was kindly donated by BASF (Florham Park, NJ). Human Caucasian Ovarian Adenocarcinoma (SKOV-3) and Rat Embryonic Cardiomyocyte (H9C2) cells were obtained from American Type

Culture Collection (Manassas, VA). Dulbecco's Modification of Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, and penicillin/streptomycin (pen/strep) were purchased from VWR (Radnor, PA). Slide-A-Lyzer™ Dialysis Cassettes, 20 K MWCO were acquired from Thermo Scientific (Rockford, IL). Cell Titer Blue® cell viability assay and Caspase-Glo 3/7® Apoptosis assay were purchased from Promega (Madison, WI). OxiSelect™ Intracellular ROS® assay was obtained from Cell Biolabs (San Diego, CA). Swiss Webster ND4 mice were procured from Harlan Laboratories (Indianapolis, IN). All other reagents used were of HPLC grade and purchased through VWR (Radnor, PA).

Methods

Preparation and characterization of RES:QUE Pluronic® F-127 micelles (mRQ)

Micelles were prepared at a RES:QUE 1:1 molar ratio using solvent casting method.[20] Briefly, RES 40 mg/mL, QUE 5.3 mg/mL, and F-127 100 mg/mL stock solutions were prepared in ethanol. Stock solutions containing 200 mg F127, 5.3 mg QUE, and 4 mg RES were combined and added to a 15 mL round bottom flask. A polymer RES-QUE film was formed using a rotoevaporator at 15 mbar vacuum pressure with the round bottom flask in contact with a 60 °C water bath. The film was formed over 8 minutes, allowed to cool to room temperature over 5 minutes, and rehydrated in 4 mL of deionized water by vortexing. The solution was sonicated for 5 minutes to ensure homogenous size distribution, and filtered using a 0.2 µm nylon filter. Micelles were characterized for size, drug loading, retention, and release.

Size of mRQ was determined by Dynamic Light Scattering (DLS) by diluting freshly prepared samples with deionized water to a final polymer concentration of 0.2 mg/mL. Samples were equilibrated for 2 minutes at 25 °C in a ZETASIZER Malvern NanoZS (Malvern, U.K.). Micelle size (diameter) is calculated by correlating the Brownian motion of the particles to the Stokes-Einstein equation at a scattering angle of 173°. The mean volume weighted size, standard deviation (SD), and polydispersity index (PDI) for three replicates is presented.

Initial loading and retention of RES and QUE in micelles was quantified by reverse-phase high performance liquid chromatography (RP-HPLC) using an Agilent 1200 Series HPLC equipped with an autosampler, binary pump, thermostatted column oven, and a DAD detector. A Zorbax SB-C18 Rapid Resolution HT column (2.1x15 mm, 1.8 micron, Agilent) was maintained at 40 °C. The mobile phase consisted of water:methanol 55%:45% stabilized with 1% methanol and 0.1% phosphoric acid. The flow rate was maintained at 0.3 mL/min and an injection volume of 3 µL was used with a 7 minute run time. RES peak area was assessed at 306 nm, and QUE at 370 nm using a DAD detector at retention times of 1.43 min and 3.29 min, respectively. Freshly prepared mRQ were diluted 1:100 in methanol and were assessed for loading at time of preparation (0 hr), and at 24, and 48 hr to determine initial loading and drug retention in the micelles at room temperature protected from light. Data is presented as mean loading ± SD for four replicates.

***In vitro* drug release from mRQ under sink conditions**

Four freshly prepared mRQ samples (3 mL each) were placed in dialysis cassettes with a MWCO of 20 K Da. The MWCO was chosen to ensure unimpeded diffusion of free RES, QUE, and F127 molecules to ensure sink conditions. The cassettes were placed in 2.0 L of 0.2 M pH 7.4 phosphate buffer, maintained at 37 °C for 48 hr. The buffer was changed every 3 hr to ensure sink conditions were maintained for the duration of the experiment. At 0, 0.5, 1, 2, 3, 6, 10.5, 24, and 48 hr samples (100 µL) were withdrawn and replaced with an equal volume of fresh buffer. Collected samples were diluted 1:100 in methanol and quantified with RP-HPLC using the method described above. The data is presented as mean percent release ± SD of four replicates. Curve fitting with first order association kinetics was completed with GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California, USA. The first order rate constants for RES, and QUE along with their calculated half-times ($t_{1/2}$) and goodness of fit (r^2) values are for four replicates are presented.

***In vitro* cell viability and apoptosis studies**

SKOV-3 and H9C2 cells were seeded at 5,000 and 10,000 cells/well respectively, and cultured in a 96-well flat bottom plate for 24 hr at 37 °C at 5% CO₂. Cells were treated with RES, QUE, ADR or RES:QUE:ADR (RQA) at 10:10:1 molar ratio in DMSO. The final concentration of DMSO in the wells was 1%. Cells were also treated with mRQ+ADR (mRQA) at 10:10:1 molar ratio diluted in normal saline. Concentrations of ADR varied from 0.001 – 100 µM and the natural products from 0.01 – 1000 µM. Upon treatment, the plates were incubated for an additional 48 hr and cell viability was assessed using CellTiter Blue[®] assay. Per manufacturer's instructions, 20 µL of the reagent was added to each well, and the cells were incubated for 2 hr. The fluorescence intensity was measured at 560_{EX}/590_{EM} nm. Data was analyzed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California, USA. Cell Viability data is presented as mean IC₅₀ ± SD of four replicates.

Combination index (CI) analysis was calculated using Compusyn software version 1.00 from ComboSyn, Inc (Paramus, NJ) by Chou and Martin.[21] Synergism and antagonism were calculated based on the Median-Effect Principle and the Combination Index-Isobologram Theorem. The CI equation used was:

$$\frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_3}{(D_x)_3} = CI.$$

Where, D stands for Drug and the subscripts 1, 2, or 3 denote the individual drug. The subscript x stands for the % inhibition in cell proliferation seen at a particular dose of the drug. CI values of < 1 indicate synergism, 1 indicate additivity, and >1 indicate antagonism. The CI can be calculated for various fractions of cells affected (fa). The utility of plotting the CI vs fa is that it provides the interaction at all affected levels (1 – 99%) for a given combination. The Mean CI vs fa data plots have been generated for each cell line and are representative of four replicates.

For the Caspase-Glo 3/7[®] assay, SKOV-3 and H9C2 cells were seeded according to the cell viability assay in opaque 96-well plates. Wells were treated with ADR, RQA, or mRQA, at

ADR concentrations between 0.001 to 1 μM . Per manufacturer's instructions, at 48 hr post-treatment, 100 μL of the reagent was added to each well in a 1:1 ratio of reagent to sample volume. After incubating the samples for 90 minutes at room temperature, luminescence was measured. Data is presented as Caspase 3/7 Activity \pm SD for four replicates.

Determination of Reactive Oxygen Species (ROS) in H9C2 cells

SKOV-3 or H9C2 cells were seeded at 20,000 cells/well in opaque 96 well plates with a 24 hour attachment time at 37 $^{\circ}\text{C}$ and 5% CO_2 . OxiSelectTM Intracellular ROS Assay was run using the manufacturer's instructions. Briefly, cells were washed with PBS without calcium and magnesium three times and incubated with 100 μL of 1 mM 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye in DMEM at 37 $^{\circ}\text{C}$ and 5% CO_2 for 1 hr. The dye was removed and cells were washed with PBS without calcium and magnesium three times. Cells were treated with either ADR, RQA, or mRQA at ADR concentrations of 0.001, 0.01, and 0.1 μM . Two hr after treatment, fluorescence was read at 480_{EX}/530_{EM} nm. Given that the concentration of the dye in the cells is 1 mM and the highest concentration of ADR is 0.1 μM any false positives generated by the ADR, which has a similar excitation and emission range, are anticipated to be far less than the signal seen with the dye. Treatments were run in quadruplicate and data is presented as mean ROS Activity \pm SD.

***In vivo* assessment of cardioprotective effect of mRQ in acute, toxic ADR dosing**

ADR is known to cause acute cardiotoxicity when dosed at 15 mg/kg or greater.[22] Therefore, we designed the animal study to induce acute cardiotoxicity by dosing at 18 mg/kg over 10 days and evaluated the cardioprotective effect of the mRQ dosed concurrently.

A mixed population of male and female Swiss Webster mice aged 12 weeks were divided into 4 groups (5 mice/group; n=20 mice) and were injected via tail vein every 3 days for 3 cycles. Mice were left untreated, injected with F127 50 mg/mL stock solution corresponding to 1.2 g F127/kg, and equivalent to the dose of F127 the mice received with mRQ formulation. ADR group was injected at 6 mg/kg ADR and mRQA group was injected at 23.6 mg/kg RES, 31.3 mg/kg QUE, and 6 mg/kg ADR. ADR formulation was prepared by dissolving the drug in normal saline for a final concentration of 12 mg/mL and sterilized by filtration using a 0.2 μM nylon filter prior to use. All injection volumes varied between 80 – 120 μL depending on the weight of the mouse to ensure all mice were getting the same dose of all compounds. Mice weight was monitored during the course of the study. On day 10, mice were euthanized and the blood was collected by cardiac puncture using heparin-rinsed 1.0 mL syringes. Plasma was separated by centrifugation at 900xg for 10 min immediately post-collection for biochemical estimations.[23] Biochemical analysis was performed for Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Creatine Kinase (CK), known markers for cardiotoxicity.[22] Compiled data is presented as mean biochemical parameter \pm SD. The biochemical analysis was performed by the Veterinary Diagnostic Laboratory in the College of Veterinary Medicine at Oregon State University. The animal work was conducted in compliance with NIH guidelines and Institutional Animal Care and Use Committee policy at Oregon State University and Pacific University for End-Stage Illness and Pre-emptive Euthanasia based on Human Endpoints Guidelines.

Statistical Analysis

Significant differences between treatment-group means for the Caspase 3/7 assay, ROS assay, and animal biochemical estimations was evaluated using one-way ANOVA with Dunnett's multiple comparisons test for equal variances, using a threshold value (α) of 0.05. Data analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California, USA.

RESULTS, DISCUSSION & CONCLUSION

Preparation and characterization of RES:QUE Pluronic® F-127 micelles (mRQ)

Micelles were prepared using a solvent casting method and were able to solubilize 1.1 mg/mL of RES and 1.42 mg/mL of QUE (Figure 1). The solubility enhancement for RES and QUE compared to their intrinsic solubility is 40 and 650 fold higher, respectively. This enhancement in solubility provided by the formulation allows us to assess these natural products at clinically relevant concentrations. RES and QUE retention in micelles was assessed over 48 hr, and the micelles were able to maintain loading for the duration of the study (Figure 1). The DLS indicated the z-average diameter for mRQ was 22.34 ± 0.15 nm with a PDI of 0.111 ± 0.001 , respectively.

Characterization of mRQ micelles

indicated stable micelles were formed and the size is consistent with previously reported literature of micelles prepared with Pluronic®.[20, 24] Further studies in our lab have indicated that these micelles are able to retain RES and QUE at these concentrations, with no change in size, for 2 weeks when stored in the dark at room temperature (data not shown).

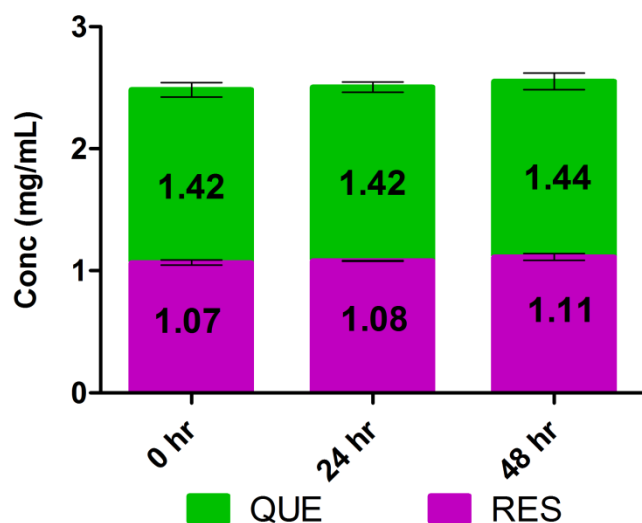


Figure 1 - Initial loading and retention of RES and QUE over 48 hr in Pluronic® F127 micelles (n =4)

In vitro drug release from mRQ under sink conditions

The release of drugs from mRQ was assessed over 48 hr at various time points under sink conditions at 37 °C and pH 7.4. Release data for RES and QUE from mRQ is shown in Figure 2. Based on the release over 48 hr, the data demonstrates approximately 70% of both RES and QUE are released from the micelles. The $t_{1/2}$ for RES in mRQ was 8.065 hr with an r^2 value of 0.9479 and for QUE 4.348 hr with an r^2 value of 0.9176. The first order rate constants for RES and QUE are 0.0859 hr^{-1} and 0.1594 hr^{-1} respectively. Based on the curve fitting, the first order association is valid indicating that drug release is diffusion driven. However, *in vivo* we anticipate that the dissociation of the micelles will occur more rapidly due to larger blood volume dilution, dynamic clearance mechanisms, and plasma protein binding. Previous studies

have indicated that F127 micelles, due to their core hydrophobicity and kinetic stability, are capable of increasing the circulating times of the encapsulated molecules.[25, 26] Therefore, while *in vitro* release kinetics do not directly correlate with *in vivo* circulating times, it is anticipated that the half-life of RES in mRQ will be greater than free RES (1.8 hr) in humans.[27] While QUE has a long terminal half-life (11 hr), its oral bioavailability (< 1 %), and aqueous solubility (2.2 µg/mL) are low.[16, 20] Intravenous micellar delivery of QUE mitigates both the solubility concerns and the oral bioavailability issues.

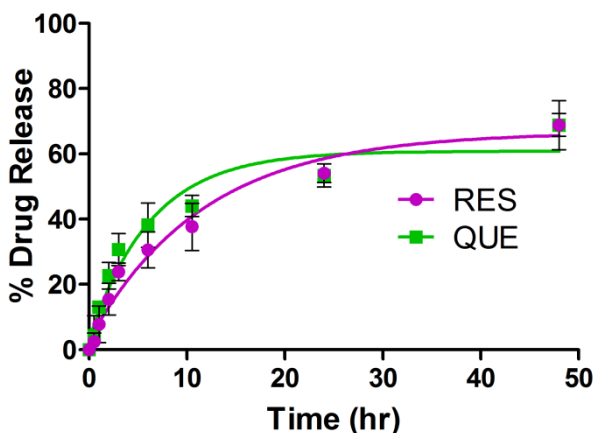


Figure 2 - *In vitro* release of RES and QUE over 48 hr from Pluronic® F127 micelles (n =4); Data was curve fitted for first-order association using GraphPad Prism

***In vitro* cell viability and apoptosis studies**

RES, QUE, ADR, RQA, and mRQA cell viability was assessed in SKOV-3 and H9C2 cells post 48 hr treatment, with the data presented in Figure 3A&C, respectively. CI values for all fa are presented in Figure 3B&D for SKOV-3 and H9C2 cell lines, respectively. As demonstrated by the data, the RQA and mRQA combinations were antagonistic (CI > 1) in H9C2 cells (Figure 3D), with a greater degree of antagonism demonstrated by the mRQA combination as compared to the RQA combination in DMSO. The antagonistic effects of RES and QUE demonstrated in H9C2 cells may be attributable to the free radical scavenging abilities of these molecules.[28] In SKOV-3 cells (Figure 3B), at low concentrations the RQA and mRQA are antagonistic, but as their concentrations increase, they become strongly synergistic (CI < 1). The results seen with RES and QUE in increasing the potency of ADR in SKOV-3 cells are in line with published data which indicate that these natural products have chemosensitizing effects when used in combination with anticancer agents.[9] The overall results demonstrate that formulation of the RES and QUE in micelles does not impede the effects of the natural products, and in the case of H9C2 cells, seem to amplify them. Control studies with blank Pluronic® micelles were also performed in our lab and these had no adverse effects on cell viability at micellar concentrations (data not shown).

As seen in Figure 3, RES:QUE:ADR combination of 10:10:1 exhibited synergistic effects in SKOV-3 cells and antagonistic effects in H9C2. Previous studies in our lab (unpublished data) with RES or QUE or other RES:QUE combinations with ADR as part of a concurrent treatment resulted in either synergistic or antagonistic effects only in both SKOV-3 and H9C2 cells. Establishing the ratio for RES:QUE: ADR capable of producing synergistic effects in SKOV-3 and antagonistic effects in H9C2 is a critical determinant for pursuing concurrent therapy with ADR as a therapeutic strategy. Therefore, all further studies were pursued at this ratio.

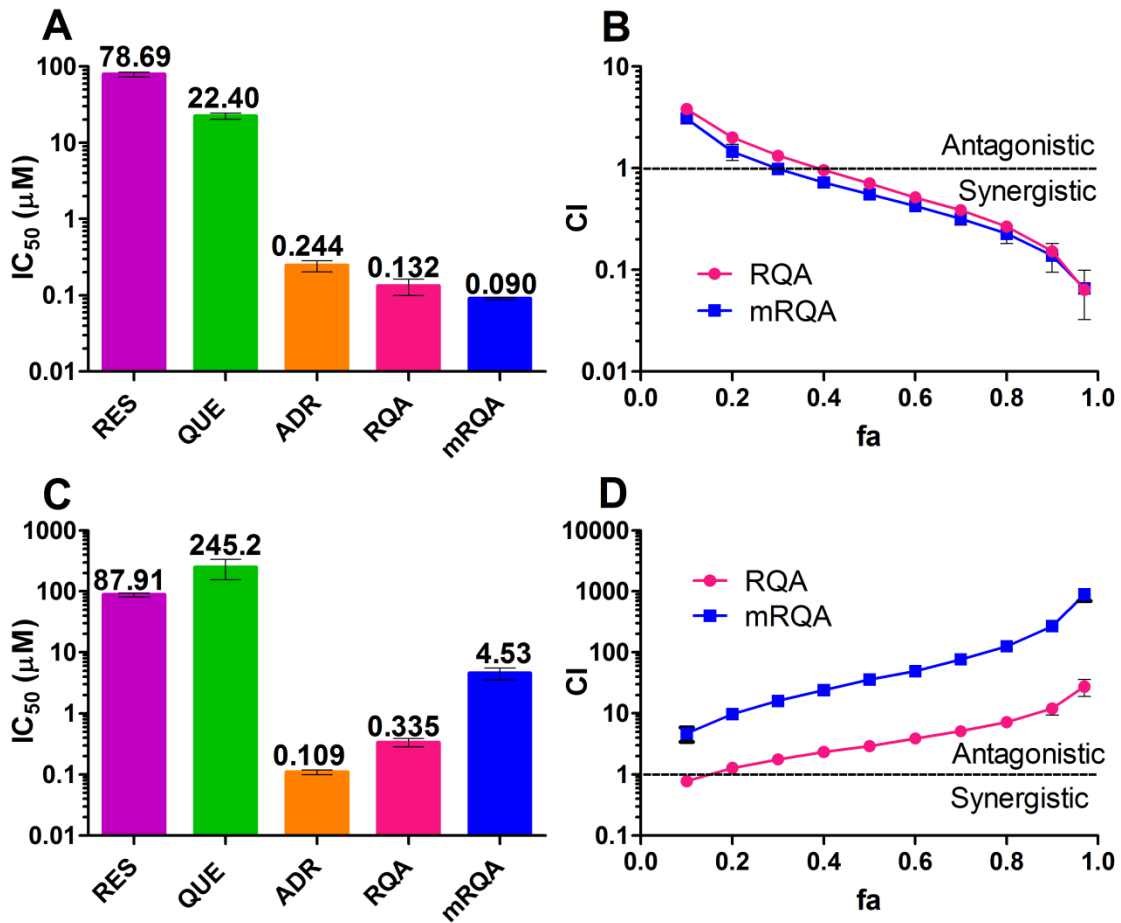


Figure 3 - IC₅₀ values of RES, QUE, ADR, RQA, and mRQA in SKOV-3 (A) and H9C2 (C) cells (n=4). CI vs fa plots for the RQA and mRQA combinations in SKOV-3 (B) and H9C2 cells (D) (n =4); CI values <1, 1, and > 1 indicate synergy, additivity, and antagonism respectively.

Many anti-cancer agents like ADR can trigger apoptosis via caspases 3 and 7 activation implicating either the intrinsic or extrinsic pathway for terminal cell death. Thus, assessing caspase 3/7 can provide information about terminal cell death irrespective of the pathway.[29] To determine whether cell death in SKOV-3 and H9C2 cells is driven by apoptosis, caspase 3/7 activity was assessed for all treatments. The cells were treated with ADR, RQA, or mRQA at ADR concentrations corresponding to 0, 0.01, 0.1 and 1 μM. The 1 μM concentration was chosen as the maximal ADR concentration, as it is at least 5 fold above the IC₅₀ value of the drug in either SKOV-3 or H9C2 cells (Figure 3A&C). The data for caspase 3/7 activity in SKOV-3 and H9C2 cells is shown in Figure 4A&B. As seen in the data (Figure 4A), in SKOV-3 cells, the fold increase in Caspase 3/7 activity for ADR, RQA, and mRQA is higher than control (untreated cells), while the RQA and mRQA groups are not statistically different in their caspase activation as compared to ADR. This indicates that the presence of the RES and QUE did not significantly decrease ADR induced caspase 3/7 activity, which is an important aspect of its antitumor efficacy. In H9C2 cells, there is a marked decrease in caspase 3/7 activity in the presence of RES and QUE at 1 μM ADR (Figure 4B), thus, supporting the CI analysis data that

the presence of RES and QUE antagonizes the effects of ADR activity in H9C2 cells. However, the caspase 3/7 activity does not return to baseline (untreated), indicating that full mitigation of ADR induced cardiotoxicity is not achieved. Figures 3 & 4 show that SKOV-3 and H9C2 cells responded to ADR in different way due to the differential genetic sensitivities between the two cell lines.

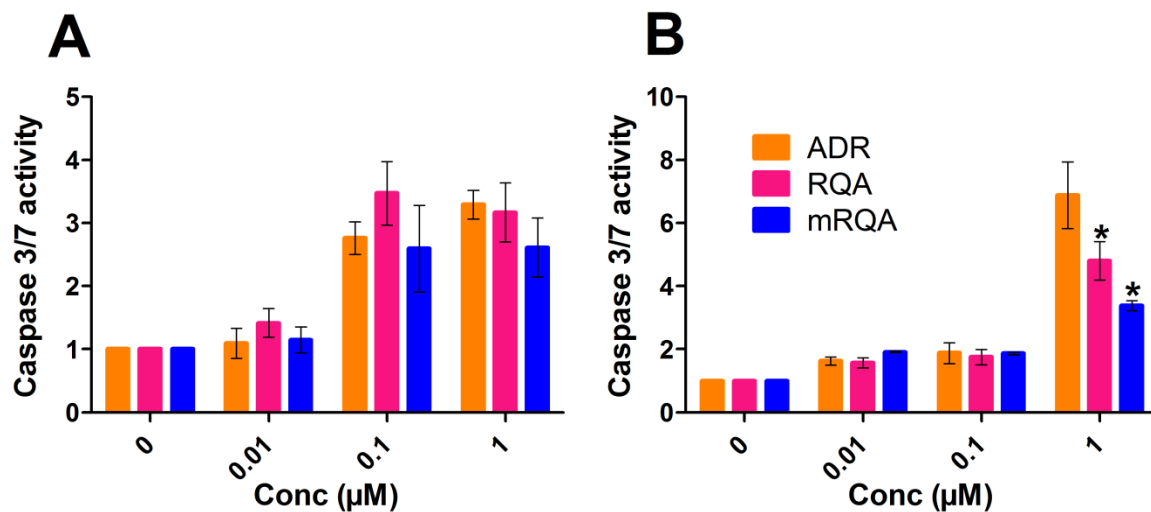


Figure 4 - Caspase 3/7 activity in SKOV-3 (A) and H9C2 (B) cells (n=4). Caspase Activity is expressed as fold increases over control (untreated) cell values. * indicates statistical significance as compared to ADR treatment alone at p-value < 0.05

Determination of Reactive Oxygen Species (ROS) in H9C2 cells

To assess the role of free radical scavenging and its effect on ADR mechanism of action in SKOV-3 and H9C2 cells, a ROS assay was run with same treatments used in the caspase 3/7 assay. The results for the change in ROS activity in the presence and absence of RES and QUE are presented in Figure 5A&B. In SKOV-3 cells (Figure 5A), the presence of RES and QUE did not significantly decrease the generation of free radicals, thus indicating that the mechanism of action of generating free radicals by ADR is preserved in the presence of these natural products. In contrast, in H9C2 cells (Figure 5B), a statistical decrease in free radical generation is seen at all concentrations of ADR from 0.01 to 1 μM in the presence of RES and QUE, Thus these results support the findings from the CI analysis and Caspase 3/7 data, in terms of protecting H9C2 cells from ADR-induced damage. ROS activity at higher RES and QUE concentrations did approach baseline values (untreated cells) and was significantly lower for both the RQA and mRQA groups as compared to ADR.

While mRQ offers significant cardioprotection in H9C2 cells, it was not complete (return to untreated levels) as demonstrated by the Caspase 3/7 activity and ROS activity in these cells. ADR has multiple mechanisms of action including inhibiting both DNA replication and RNA transcription, generating free radicals, which leads to DNA damage or lipid peroxidation, crosslinking DNA, alkylating DNA, and causing direct membrane damage due to lipid oxidation

and inhibiting topoisomerase II.[1] ADR induction of cardiotoxicity is also postulated to be multifactorial with free radical generation and lipid peroxidation playing an important role. However, other proposed mechanisms for ADR induced toxicity include the inhibition of nucleic acid and protein synthesis, release of vasoactive amines, changes in adrenergic function, abnormalities in the mitochondria, lysosomal alterations, altered sarcolemmal Ca^{2+} transport, changes in adenylate cyclase, Na^+ , K^+ ATPase and Ca^{2+} ATPase, imbalance in myocardial electrolytes, and depletion of non-protein tissue sulfhydryl compounds.[30, 31] Recently, studies have shown that deletion of the gene encoding for topoisomerase-II β (Top2b) in cardiomyocytes in mice protects them from ADR induced cardiotoxicity.[32] Thus, due to the nature of the multifactorial process of cardiotoxicity it is possible that free radical scavenging alone cannot fully mitigate it. Therefore, *in vivo* studies are a critical component of evaluating this therapeutic strategy as seen in the next section.

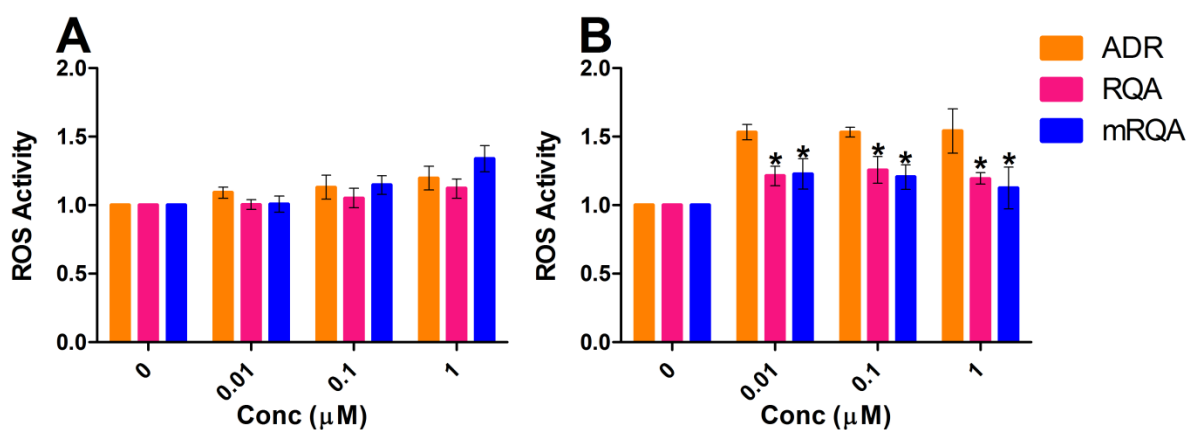


Figure 5 - ROS activity in SKOV-3 (A) and H9C2 (B) cells (n=4). ROS Activity is expressed as fold increases over control (untreated) cell values. * indicates statistical significance as compared to ADR treatment alone at p-value < 0.05

The differential effects seen in the “normal” (H9C2) versus the cancerous (SKOV-3) cells generation of free radicals in the presence of the phytochemicals (Figure 5) is a new finding that has not been reported elsewhere. While many of these phytochemicals are implicated in chemoprevention and more specifically through free radical scavenging, similar studies have not been undertaken in existing cancer tissue.[33] The interplay between the chemosensitization effects and the free radical scavenging effects in cancer lines requires further mechanistic studies. As seen in figures 3 (B&D) the degree of synergisms and antagonism are a function of ADR and natural product concentration, and at higher concentrations the synergism (chemosensitization) seems to win out on SKOV-3 cells while the antioxidant (antagonistic) effects become more prominent in H9C2 cells.

***In vivo* assessment of cardioprotective effect of mRQ in acute, toxic ADR dosing**

In vivo biochemical estimation data in mixed population Swiss Webster mice is presented in Figure 6. Mice were left untreated (Untx mice), or treated with vehicle (F127), ADR, or mRQA at concentrations known to induce acute cardiotoxicity in mice. AST, ALT, and CK are all known surrogate markers for cardiotoxicity.[34] As expected, at high doses of ADR, the AST, ALT, and CK levels were significantly higher compared to untreated mice (Figure 6). In

contrast, the levels of AST, ALT, and CK in mRQA group were similar to untreated mice, indicating that the concurrent treatment of RES and QUE at 10:10:1 ratio with ADR is a viable approach for short-term cardioprotection in acute ADR dosing. Thus, indicating the feasibility of this strategy in fully mitigating ADR induced cardiotoxicity. However, future studies need to be conducted for longer term determination of cardioprotection as part of a treatment regimen to establish the full utility of this approach. It is also possible that combining mRQ and ADR with another moiety that differentially affects Top2A may provide a more complete cardioprotection.

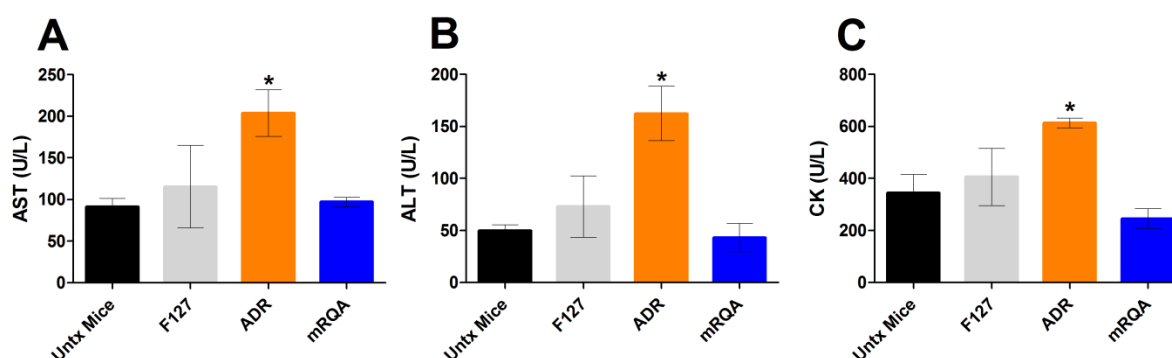


Figure 6 - Biochemical estimations from mice treated with acute doses of ADR with or without mRQ and controls (untreated or vehicle (F127) treated); (A) AST, (B) ALT, and (C) CK (n=5). * indicates statistical significance as compared to untreated mice at p-value < 0.05

In conclusion, we have designed and characterized a polymeric micellar based natural product adjuvant (mRQ) for the mitigation of ADR induced cardiotoxicity which is efficacious both *in vitro* and *in vivo*. In addition, preliminary *in vitro* studies in a human ovarian cancer cell line has indicated that this approach might provide a secondary benefit of acting as a chemosensitizer in these cells, further enhancing the potential of this therapeutic approach.

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