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Influence of controlled release of resveratrol from electrospun fibers in combination with siRNA on leukemia cells

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ABSTRACT

In this study, we evaluated the possibility of i) local release of resveratrol from poly(ε-caprolactone) (PCL) and gelatin (GT) electrospun fibers and ii) combining (i) with siRNA designed to downregulate BCR-ABL pathway on K562 cancer cells. Initially, K562 cell culture experiments were performed using various bolus doses of resveratrol in combination with siRNA for 3 days using a factorial design of experiments approach. Resveratrol content was analyzed using HPLC and cell viability was assessed using Annexin V (Non-viable), and Propidium Iodide (PI) (Necrotic) based flow cytometry. Coaxial electrospun fibers with resveratrol were made using 1:1 PCL-GT blends in different configurations: single fibers and coaxial fibers with same polymer blends, or with PCL inner core. Loading efficiency and release profile over five days were analyzed. Based on release profile, K562 cell viability with fibers was analyzed over eight days. Dose dependent cell death was observed with bolus resveratrol and siRNA in the culture. However, resveratrol content depleted significantly when added directly to solution. The combination therapy was additive in solution. SEM analysis showed no phase separation of components and resveratrol loading efficiency varied from 77% to 88% in different configurations; 95% of resveratrol was released by day five. Permeability of resveratrol showed no significant dependency on fiber configuration. After 8 days, non-viable cell percentages with controlled release were similar to that at three-day bolus dose of resveratrol. However, siRNA interacted with the fibers, resulting in reduced effect on cells. Loading resveratrol into electrospun fibers provides a localized delivery at therapeutic level, and increased resveratrol's apoptotic effect. Using single fibers is sufficient for controlled release.

1. Introduction

Resveratrol, a polyphenolic compound present in red wine, grapes, peanuts, fruits, mulberries, and pines, has gained significant attention. In the early 1990s, consumption of red wine in France was attributed to the low incidence of cardiovascular diseases, despite higher consumption of saturated fat (Renaud and de Lorgeril, 1992). This was termed “French paradox”, and provided significant impetus to exploring resveratrol as a dietary supplement in cardiac and cancer therapies (Kulkarni and Canto, 2015; Novelle et al., 2015). Molecular mechanisms through which resveratrol mediate such interactions is explored in various disease models, including cancer (Kundu and Surh, 2008). In vitro studies show that resveratrol induces apoptosis via Sphingosine kinase 1 (SphK1) inhibition, which is associated in promoting various types of cancers including chronic myeloid leukemia (CML) (Tian and Yu, 2015), and prostate cancer (Brizuela et al., 2010). Others have shown that resveratrol causes autophagy in K562 cells, the erythroleukemia cell type commonly used to study CML (Koeffler and Golde, 1980) and formation of intracellular autophagosomes (Puissant et al., 2010). Resveratrol is shown to cause changes in mitochondrial activity (Prabhu et al., 2013) using tetrazolium salt-based calorimetric assays, which is reduced to formazan.

Clinical trials performed by administering 500 mg of trans-resveratrol showed no significant side effects (Tomé-Carneiro et al., 2013). Another study with 150 mg/day of trans-resveratrol given to healthy volunteers showed signs of calorie restricted diet effects (Timmers et al., 2011). However, plasma levels after 30 days were in nanomolar range, nearly 3 orders of magnitude less than required to cause apoptosis in cancer cells in vitro. Rapid reduction in bioavailability of resveratrol after systemic administration is attributed to various factors, including biotransformation, short half-life ($1-3$ h), rapid uptake by cells, and lipophilic characteristics with limited solubility in water (Almeida et al., 2009; Sergides et al., 2016). Metabolism of resveratrol also varies from person to person due to alterations in gut-associated bacteria (Bode et al., 2013). In order to obtain therapeutic levels needed for inducing apoptosis, controlled release of trans-resveratrol at the site

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of interest is necessary. In addition, combining resveratrol with other drugs targeting other cellular pathways could reduce the dosage requirements (Kim et al., 2014; Vendrely et al., 2017).

Recently, electrospun fibers have emerged as ideal candidates in drug delivery systems, as they offer a better degree of control over the release kinetics relative to competing methods such as nanoparticles (Kumari et al., 2010). In particular, formation of multilayered core-shell fibers using multiaxial electrospinning allows sequestering stimulants in different compartments to modulate the release kinetics (Khalf and Madihally, 2017b). Also, one could blend lipophilic polymers, such as poly(ε-caprolactone) (PCL), and hydrophilic polymers, such as gelatin (GT), in various ratios and form fibers. These combinations allow encapsulation of both hydrophilic and lipophilic (or hydrophobic) drugs while providing high surface to volume ratio, cell attachment, and drug loading. These polymers are also biocompatible and biodegradable i.e., they can be local implanted with the expectation of complete degradation. However, there is a lack of model-based fundamental understanding of influence of such layering and polymer selection on the permeability of lipophilic drugs.

In this regard, we first tested the combination effect of resveratrol and small interfering RNA (siRNA), to downregulate the fused breakpoint cluster region (BCR)-Abelson (ABL) tyrosine kinase gene pathway; BCR-ABL deregulation is shown to be sufficient to sustain CML phenotype (Deininger et al., 2000; Heisterkamp et al., 1990). Based on the literature reports, resveratrol (Tian and Yu, 2015) and siRNA (Valencia-Serna et al., 2013) dosage were selected. Combination of resveratrol and siRNA was more effective in inducing apoptosis even at lower doses. Next, we selected 40 μM resveratrol and formed PCL-GT hybrid coaxial fibers using a common solvent and a previously published method (Khalf and Madihally, 2017a). We evaluated the release profile of resveratrol and its effect on K562 cells. Loading resveratrol into electrospun fibers provided a delivery method needed to obtain dosage levels locally. Evaluation of permeability using resistance in series model showed no dependency on layering, suggesting that rate of lipophilic drug release is less dependent on various layers.

2. Materials and methods

2.1. Materials

PCL (80 kDa, Mn = 80,000), Type A gelatin (porcine 300 Bloom), 2,2,2-trifluoroethanol (TFE), Caffeine, propidium iodide (PI) powder, sterile dimethyl sulfoxide (DMSO), and trans-resveratrol were purchased from Sigma-Aldrich (St. Louis, USA). A custom-synthesized BCR-ABL siRNA (5′-GCAGAGUUCAAAAGCCCTT-3′), and a corresponding scrambled siRNA (5′-GCCCCAAGATATAGGTTCA-3′) were purchased from Integrated DNA Technologies (Coralville, IA). Annexin V FITC conjugate was purchased from Thermo Fisher Scientific. K562 cells were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, USA), 2 mM Glutamine, prepared with 10% FBS obtained from ATCC (Manassas, VA).

2.2. Cell culture maintenance

K562 cells were cultured in T75 cell culture flasks following vendors protocol. In brief, cells were incubated in 5% CO_2/95% air, at 37 °C and culture medium was changed every 3 days by centrifuging cell suspensions for 5 min at 840 × *g* and 4 °C. The formed cell pellet was resuspended in 9 mL of fresh culture medium and plated in a fresh T75 flask. All cultures were routinely monitored under an Evos™ AME-i2111 Digital Inverted Microscope, and when necessary, phase contrast micrographs were obtained at random locations.

2.3. Resveratrol and siRNA dosage effect in solution

For all experiments, cells were harvested, counted using the hemocytometer, and 2×10^5 cells/mL were cultured in a 6-well plate with 2 mL of fresh media for each well. All cell cultures were incubated for 72 h prior to analysis for viability as described in cell viability section.

2.3.1. Resveratrol alone

A 40 mM stock solution of resveratrol was dissolved in DMSO and stored at 4 °C until further use. Based on a previously published report (Tian and Yu, 2015), samples were prepared with 0, 10, 20, 40, 80, and 160 μM resveratrol concentrations. Zero concentration condition had DMSO equivalent to that present in 160 μM resveratrol condition, added directly.

2.3.2. siRNA alone

A stock solution of 625 nM siRNA in 150 mM NaCl was prepared. Based on a previously published report (Valencia-Serna et al., 2013), samples were prepared with 0, 12, 24, 36, 48, and 60 nM range of siRNA concentration. Zero concentration condition contained NaCl equivalent to that present in 60 nM siRNA condition, was added directly. Scrambled siRNA was tested at 36 nM concentration.

2.3.3. Resveratrol and siRNA combination experiments

Three conditions were used based on a factorial design of experimental approach:

i). 40 μM resveratrol and 36 nM siRNA.
ii). Mid-point concentrations using 20 μM resveratrol, and 18 nM siRNA.
iii). Control samples were prepared by directly adding DMSO and NaCl solution equivalent to that present in 40 μM resveratrol solution and 36 nM siRNA solution.

2.4. Cell viability analysis

After various incubation times, cells were centrifuged at 840 × *g* for 5 min, and washed with phosphate buffer solution (PBS, 7.2 pH, prepared in-house using 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 2.17 g Na_2HPO_4-$7H_2O$ in 975 mL DI water) with 0.1% BSA (1 mL for every 2×10^5 cells). Then cells were stained with Annexin V buffer, using vendor's protocol. In brief, cells were centrifuged again and washed with Annexin V buffer solution (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl_2$ and 10 mM HEPES in DI water). Cells were centrifuged and resuspended in Annexin V buffer solution (50 μL per 10^5 cells). Then, 1 μL per 10^5 cells of Annexin V FITC conjugate were added and incubated in ice for 15 min. Also, 50 μL per 10^5 cells of Annexin V buffer were added, followed by 4 μL of PI (100 mg/L), and then incubated in ice for another 15 min. Cells were washed with 500 μL of Annexin V buffer solution, and the suspension was then discarded. The cells pellet was re-suspended in 500 μL of Annexin V buffer solution, and incubated on ice for 10 min before samples were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer. Unstained samples and individually stained samples were used as controls. Obtained data for each sample was plotted in dot plots, where the cell populations separate into at least two groups: live cells with a low level of fluorescence and non-viable cells with a substantially higher fluorescence intensity. Dead cells were labeled with both the PI and Annexin V conjugate. Based on this information, percentages of dead cells were obtained using a standard quadrant analysis.

2.5. Stability of resveratrol in culture medium

In order to understand the changes in resveratrol concentration during 3-day incubation, cell culture experiments were performed in 6-well plates in two groups: i) with 2×10^5 K562 cells/mL cells and ii) without cells, but with the same amount of cell culture medium. In both groups, 40 μM resveratrol was added to the culture medium using the