Targeting Chemotherapy Resistance in Non-Small Cell Lung Cancer via Berry Anthocyanidins

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Introduction

- Lung cancer is the leading cause of cancer deaths in the United States and the world.
- Multidrug resistance (MDR) is one of the main factors leading to the failure of chemotherapy, targeted therapy, and immunotherapy.
- Paclitaxel (PAC) is a leading chemotherapy for aggressive lung cancer. Drug resistance, specifically PAC resistance, thus plays an important role in the prevalence and mortality of lung cancer.
- The MDR gene encodes the membrane transporter multi-drug resistance protein 1 (MDR-1), which works through an ATP dependent efflux mechanism. Lung cancer cells respond to cancer therapies, such as PAC, by overexpressing MDR-1 as a protection mechanism.
- Our lab has previously shown that native mixture of anthocyanidins (Anthos) isolated from blueberry, in combination with PAC, had significantly enhanced cell death in drug-sensitive lung cancer cells both *in vitro* and *in vivo*. The effects were even greater when Anthos was loaded onto milk exosomes (Aqil et al. Food & Function, 2017).
- Exosomes (Fig. 1), are endogenous nanovesicles that have been suggested as a potential drug carrier. Because of their nano size (30-100 nm), exosomes can readily enter cells and deliver their payloads. Exosomes could also be functionalized with tumor-recognizing ligands such as folic acid (FA) for targetability (Aqil et al. AAPS, 2017).
- There remains an urgent need to find a way to chemo-sensitize acquired resistance and potentially prevent resistance from occurring altogether in lung cancer therapy.
- In this study, we explored if the use of the efficacious plant therapeutics (e.g., Anthos), in combination with chemo drugs (e.g., PAC), will enhance therapeutic response to overcome drug resistance in PAC-resistant lung cancer cells.
- We further evaluated the effect of Anthos and its exosomal formulation on the drug resistance protein (MDR-1) and if its downregulation could sensitize the drug-resistant cells to PAC.

Hypothesis

- We hypothesize that Anthos and PAC + Anthos will promote cell death in PAC-resistant lung cancer cells.
- Furthermore, we hypothesize that the nano-formulation of Anthos will have greater efficacy at downregulating the MDR-1 protein compared to its free agent.

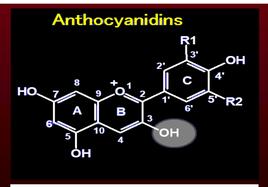

Fig. 1. General Structure of anthocyanidins (Anthos)

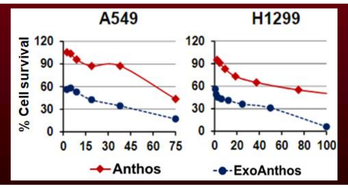

Fig. 2. MTT Assay for Anthos and Exo-Anthos in drug-sensitive lung cancer cells (Kausar and Gupta, unpublished data).

Results

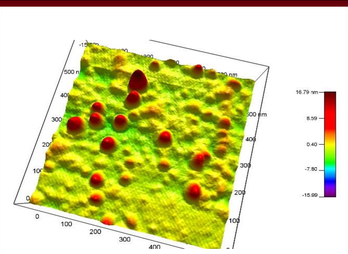

Fig. 3. Atomic Force Microscopy of milk exosomes.

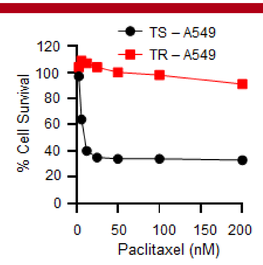

Fig. 4. MTT Assay for PAC in drug-sensitive and drug-resistant lung cancer cells.

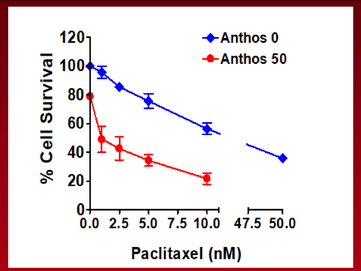

Fig. 5A. MTT Assay for Anthos and Anthos +PAC in drug-sensitive A549 lung cancer cells.

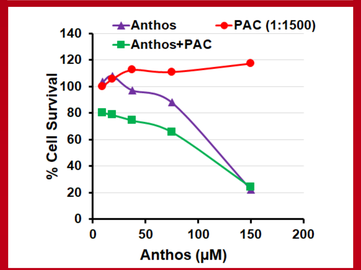

Fig. 5B. MTT Assay for Anthos, PAC, and Anthos+PAC in TR-A549 lung cancer cells.

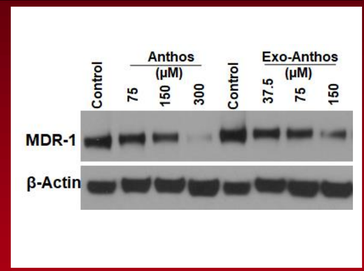

Fig. 6A. Western blot analysis of the effect of Anthos and Exo-Anthos onMDR-1 in TR-A549 lung cancer cells.

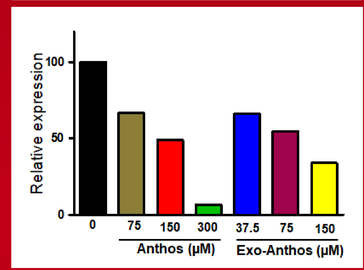

Fig. 6B. Modulation of MDR-1 by Anthos and Exo-Anthos.

Methods & Materials

Preparation of Exo-Anthos: Bovine milk-derived exosomes, Anthos and Exo-Anthos were gifts from Mr. Jeyaprakash Jeyabalan of 3P Biotechnologies, Inc. Briefly, exosomes were isolated from bovine milk through differential centrifugation and suspended in PBS. Native mixture of Anthos, isolated from bilberry, was dissolved in a mixture of acetonitrile: ethanol (1:1 v/v). Anthos solution was then added to the exosomes by simple mixing. The mixture was kept at room temperature for 15 min, followed by low-speed centrifugation to remove unbound Anthos. Anthos-loaded exosomes were then collected by high-speed centrifugation.

Exosome Characterization: Atomic force microscopy (AFM) was utilized to determine surface characteristics and size of exosomes (AFM picture was provided by Mr. Jeyaprakash Jeyabalan of 3P Biotechnologies, Inc.).

Drug Loading Analysis: Anthos loading onto exosomes was determined by ethanol precipitation of exosomal proteins. The Anthos were measured by HPLC while the protein concentrations were measured by BCA method spectrophotometrically. Percent Anthos loading was calculated by dividing the amount of Anthos with protein X 100.

Cell Culture: Cell line for PAC resistant lung cancer (TR-A549) was cultured in RPMI media with 10% (v/v) heat-inactivated fetal bovine serum and 1% antibiotics (penicillin/streptomycin), at 37°C in a humidified atmosphere of 5% CO2.

MTT Assay: Antiproliferative activity of Anthos, PAC, and Anthos + PAC against drug-sensitive (TS-A549) and drug-resistant lung cancer cells (TR-A549) was assessed by MTT assay.

Protein Concentration: Whole cell lysates were prepared using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentration of the cell lysates was measured using a BCA kit.

Western Blot Analysis: Lung cancer cell line (TR-A549) was treated with or without varying concentrations of Anthos and Exo-Anthos for 48 h. Sample lysates were separated using SDS-PAGE gel electrophoresis. After transfer, membranes were immunoblotted using primary monoclonal antibody of MDR-1 and secondary anti-mouse HRP. The transferred proteins were visualized with enhanced chemiluminescence detection kits. Protein bands were detected on X-ray film and quantified by ImageJ software. B-actin was used as a loading control.

Acknowledgements

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Summary of Findings

- Exosomal formulation of Anthos showed significantly higher growth inhibition versus the free Anthos against A549 lung cancer cells; the exosomal delivery of Anthos increased the drug efficacy (a 20-fold reduction in IC_{50}) (data not shown).
- Exosomes isolated by differential centrifugation process resulted in average particle size of about 75nm.
- The exosomes were characterized by i) AFM, and ii) presence of surface markers (CD63 and CD81) (not shown).
- Anthos loading efficacy was found to be about 7%.
- Drug-sensitive lung cancer responds well to PAC treatment with an IC_{50} of 5.4 nM, whereas drug-resistant lung cancer has little or no response at all to PAC treatment.
- The Anthos enhanced the potency of PAC in drug-sensitive lung cancer cells when treated as combination.
- The Anthos revived the activity of PAC in drug-resistant lung cancer cells when treated as combination.
- Anthos + PAC had a 20% decrease in IC_{50} compared to Anthos alone in drug-resistant lung cancer cells.
- Both Anthos and Exo-Anthos showed a significant dose-dependent inhibition of MDR-1 in drug-resistant lung cancer cell line TR-A549. However, the effect was enhanced modestly with exosomal formulation.

Conclusions

- Both Anthos and Exo-Anthos appear to chemo-sensitize PAC resistant lung cancer cells through MDR-1 downregulation.
- Exo-Anthos showed greater efficacy at lower doses, providing a non-toxic drug with tumor targetability.
- Clinical Impact: Because Exo-Anthos has shown to downregulate the MDR-1 protein at greater efficacy, it could potentially be developed as an adjuvant therapy during cancer treatment to prevent or delay acquired resistance to chemotherapy drugs.

Future Direction

- To further examine the influence of Anthos in detail on the MDR-1 mechanism.
- To evaluate the effect of Anthos + PAC on MDR-1 expression in drug-resistant lung cancer cells.
- To validate the antiproliferative and possible chemo sensitization effects of Anthos/Exo-Anthos through MDR-1 inhibition in drug-resistant lung cancer animal models.

References

1. Aqil F, Jeyabalab J, Agrawal AK, Kyakulaga AH, Munagala R, Paarker L, Gupta RC. Food & Function. 2017, 8: 4100-4107
2. Aqil F, Munagala R, Jeyabalan J, Agrawal AK, Gupta RC. AAPS. 2017, 19(6): 1691–1702.