Role of Anthocyanidins on Immune Checkpoint Protein, PD-L1 in HCT116 and HT-29 Colorectal Cancer Cells

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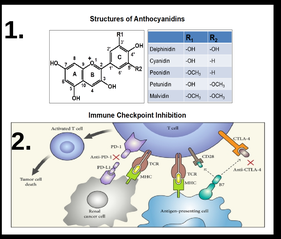
Introduction

- Colorectal cancer is the third leading cause of death effecting 4.5% of the men and 4.2% of women across the U.S.
- One of the leading factors in the influx of these numbers is immune checkpoint inhibition.
- Immune checkpoint inhibitors are proteins that block immune systemic cells such as neutrophils, macrophages, B-cells, T-cells and natural killer cells from attacking the cancer cells.
- Examples of these checkpoint inhibitors are PD-L1, PD-1, and CTLA-4. These proteins can be both inhibitory and stimulatory. PD-L1 when overexpressed act as a signal on cancerous cells telling the immune system “don’t eat me up”.
- Immunotherapy against various cancers is presently at the center stage.
- The immunotherapy uses monoclonal antibodies (pembrolizumab, Nivolumab, Atezolizumab, avelumab, durvalumab) to blockade proteins such as PD-L1 and stop them from inhibiting immune cells.
- Besides the high costs, these monoclonal antibodies pose a threat because of their high toxicity.
- Ourlaboratory has demonstrated that berry anthocyanidins (Anthos) can inhibit colon cancer, breast cancer, lung cancer and ovarian cancer in rodent models. The therapeutic activity is enhanced by embedding Anthos in milk-derived exosomes (Munagala *et al*. 2016 & 2017).
- In this study, we explored if the inhibition of colon cancer by Anthos (Figure 1) and Exo-Anthos could be, in part, due to immune checkpoint inhibition (Figure 2)

Hypothesis

* We hypothesize that the known anti-cancer activity of the Anthos is, in part, due to the inhibition of immune checkpoint proteins.

1.



- **Figure 1.** Anthocyanidins structures. (Aqil et al., 2017)
- **Figure 2.** Presentation of immune checkpoint inhibition. (Raman et al., 2015)

Results

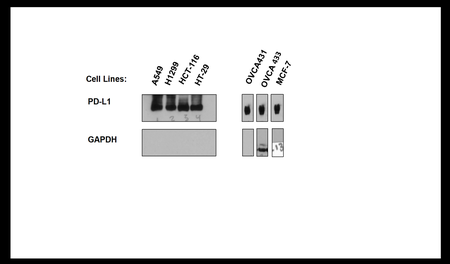


Figure 3A. Western blot analysis of PD-L1 expression In different cell lines.

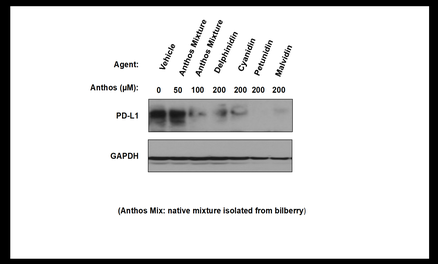


Figure 4A. Western blot analysis of the effect of Anthos mixture and individual Anthos in HT-29 colon cancer cells.

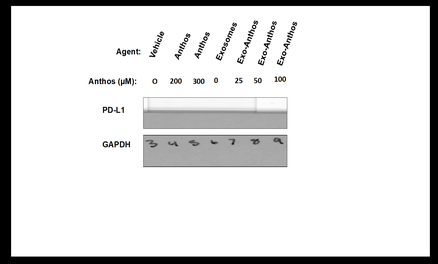


Figure 5A. Western blot analysis of the effect of Anthos and Exosomal-Anthos on HCT-116 colon cancer cells.

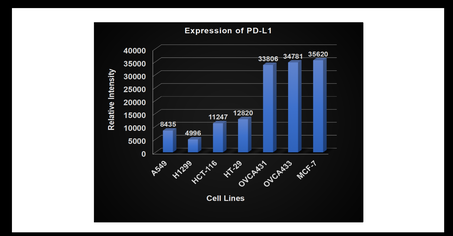


Figure 3B. Graphical representation of PD-L1 expression in different cell lines.

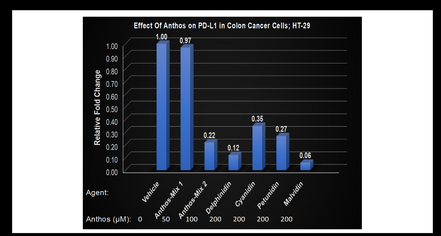


Figure 4B. Modulation of PD-L1 by Anthos mixture and individual Anthos in HT-29 colon cancer cells.

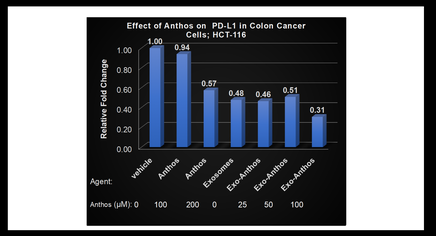


Figure 5B. Modulation of PD-L1 expression in HT-29 colon cancer cells by Anthos and Exosomal-Anthos.

Methods & Materials

Cell culture: Cell lines for ovarian cancer (OVCA 2780, OVCA 431, OVCA 433), breast cancer (MCF-7), lung cancer (A549), and colon cancer (HT-29, HCT-116) were cultured in their respective media with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), at 37°C in a humidified atmosphere of 5% CO2.

Protein concentration: Protein concentration of the cell lysates was measured using a BCA kit.

Western Blot Analysis: Colon cancer cell lines (HCT-116 and HT-29) were treated with or without with individual anthocyanidins, Anthos and Exo-Anthos for up to 48 h. Whole cell lysates (WCL) were prepared using RIPA buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Sample lysates were separated using SDS-PAGE gel. After transfer, membranes were immunoblotted using primary monoclonal antibody of PD-L1 and secondary anti-rabbit HRP (Cell Signaling, Danvers, Massachusetts). The transferred proteins were visualized with an enhanced chemiluminescence detection kits (Amersham, Piscataway, New Jersey) Protein bands were detected on x-ray film, and quantified by Image j software. GAPDH was used as a loading control.

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Summary of Findings

- Ovarian cancer (OVCA 2780), breast cancer (MCF-7) and colon cancer (HCT-116 and HT-29) showed highest expression of the immune checkpoint protein, PD-L1.
- Anthos isolated from bilberry showed a dose-dependent inhibition of PD-L1 in colon cancer cell lines, HCT-116 & HT-29; the inhibition was more prominent in HT-29.
- Individual anthocyanidins inhibited PD-L1 in the following descending order: malvidin (Mv), delphinidin (Dp), petunidin (Pt), and cyanidin (Cy).
- Milk-derived exosomes per se (in the absence of Anthos) also inhibited of PD-L1, suggesting the presence of some immune factors in milk exosomes.

Conclusion

- The data show that the anthocyanidins and milk exosomes inhibit the immune checkpoint protein, PD-L1, and suggest that the known therapeutic effect of the Anthos and the exosomes may, in part, be related to immune-boosting property of these agents.
- ‘Clinical Impact: This research finding has a potential to influence cancer treatment by offering a new form of immunotherapy for colon cancer by berry bioactives (Anthos) and the milk exosomes/exosomal formulations that are non-toxic and cost effective.

Future Direction

- To examine the effect of anthocyanidins in detail on PD-1/PD-L1 immune checkpoint pathway.
- To validate anti-cancer effect anthocyanidins by checkpoint protein inhibition in colon cancer animal models

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