Pranlukast's Role in Thiol Isomerase Inhibition and Application in Cancer-Induced Thrombosis

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Background

Cancer patients who are receiving chemotherapy treatment have a heightened risk of thrombosis, and one in ten patients, on average, will suffer from a blood clot [1,2]. Malignant cancer cells have been shown to cause higher levels of thiol isomerase enzyme activity, and thiol isomerase enzymes are composed of the endoplasmic reticulum proteins (ERp), specifically ERp5, ERp57, ERp72, and protein disulfide isomerase (PDI). Direct inhibition of these enzymes has shown promise in cancer-induced thrombosis therapy, and this study aimed to test the effectiveness of the possible thiol isomerase inhibitor, pranlukast (PRAN), which is part of a family of leukotriene receptor antagonists [3]. Other drugs in this family, including zafirlukast (ZAF) and montelukast (MONTE), are proven inhibitors of thiol isomerase activity, and ZAF has additionally been shown to decrease tumor size in OVCAR8 xenograft mice [4,5].

In this study, we examined the potential of PRAN as a possible thiol isomerase inhibitor compared to the already studied ZAF and MONTE.

Methods

Insulin assay: Each drug was diluted in a 5-point dose cure and mixed with phosphate buffer (PO_4) in a 384-well plate. Next, a mixture of PO_4, insulin, Ethylenediaminetetraacetic acid (EDTA), PDI or ERp57, and dithiothreitol (DTT) was added to the pre-specified well. The plate was then read at 650 nm for 90 minutes.

Presto Blue Assay: 10,000 human ovarian cancer cells (OVCAR8) were plated and incubated for 24 hours. Then, a specified concentration of PRAN or ZAF was added to each column and again incubated for 24 hours. On day 3, 10 µL of PrestoBlue reagent was added to each well, incubated for 15 mins, and then measured at 560/590 nm every 5 minutes for 15 minutes.

Factor Xa Generation: 100,000 OVCAR8 cells were plated and allowed to grow overnight, then washed and media replaced with TBS. The cells were then incubated with PRAN or ZAF for 30 minutes. After incubation, calcium, a fluorescent substrate, factor X, and factor VIIa were added to each well and fluorescence measured at 352/470 nm for 45 minutes.

P-selectin Flow Cytometry: ZAF or PRAN was diluted to the desired concentrations with 1% DMSO in a Tyrode's buffer. Then 5 µL of drug or vehicle control was added to the appropriate tube. Next, 37 µL of HEPES buffered saline, 1 µL of a PE conjugated P-selectin antibody, and 2 µL of platelet-rich plasma were added to each tube and allowed to incubate at room temperature for 30 minutes in the dark. Then, 5 µL collagen related peptide was added to each tube and left to incubate for 30 mins in the dark at room temperature. After incubation, 100 µL of 0.2% paraformaldehyde was added to fix the samples, which were then analyzed on a BD Accuri C6 Plus flow cytometer with 10,000 gated events recorded.

Statistics: Data were presented as the mean ±SD. A one-way ANOVA with a post hoc Dunnett's test was used to evaluate the statistical significance between test groups and the control. *P < 0.05, **P < 0.01, ***P < 0.001, or ****P <0.0001 was considered to be statistically significant.

Results

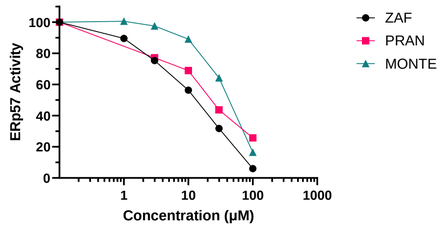

Figure 1: Insulin assays were performed to measure the inhibition of PRAN, ZAF and MONTE against the thiol isomerase ERp57. All three inhibit ERp57 with ZAF being the most potent, then PRAN then MONTE.

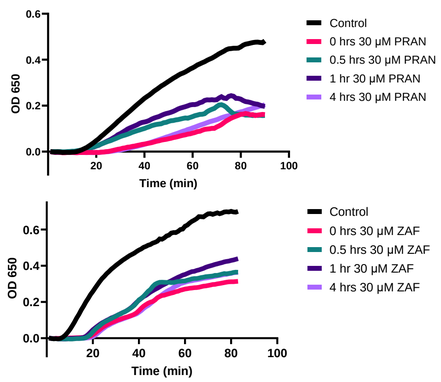

Figure 2: A modified insulin assay was performed to determine the reversibility of PRAN **(A)** and ZAF **(B).** Neither drug shows a pre-incubation time dependance suggesting both ZAF and PRAN are reversible thiol isomerase inhibitors.

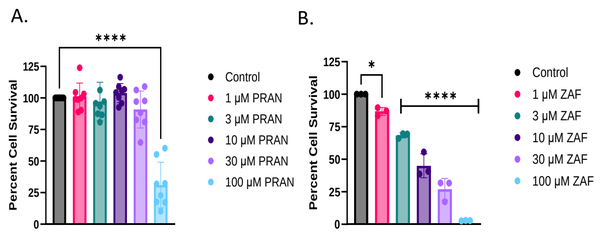

Figure 3: OVCAR8 cell survival of PRAN **(A)** and ZAF **(B).** ZAF consistently outperformed PRAN in cell destruction, with adjusted p-values consistently showing statistical significance, while only 100 µM showed statistical significance for PRAN.

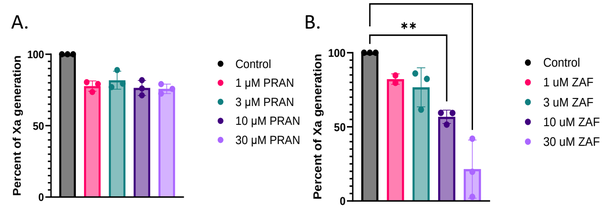

Figure 4: PRAN **(A)** and ZAF **(B)** inhibit factor Xa generation. PRAN consistently inhibited factor Xa generation at about 25% less than the control. ZAF inhibited Xa generation in a dose-dependent manner at 1, 3, 10, and 30 µM, with 30 µM reaching about 78% inhibition.

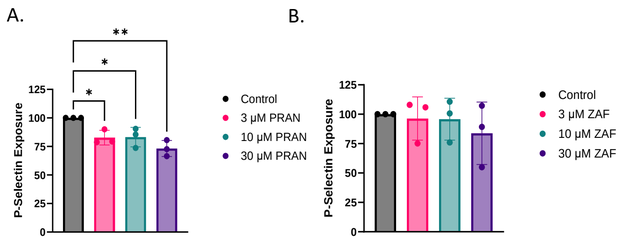

Figure 5: P-selectin exposure was measured for PRAN **(A)** and ZAF **(B)** at 3, 10, and 30 µM**.** PRAN was slightly better than ZAF at inhibiting P-selectin exposure, with PRAN demonstrating 30% inhibition at 30 µM**.**

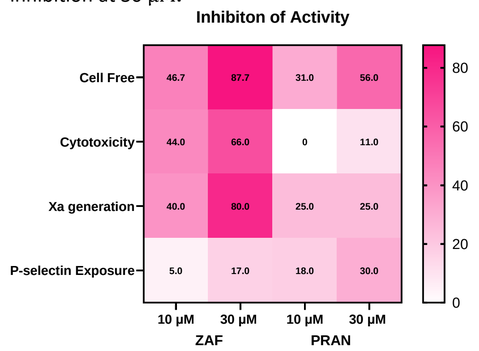

Figure 6: ZAF and PRAN were compared in the various assays performed throughout this study. ZAF consistently outperformed PRAN at both 10 µM and 30 µM throughout cell free, cytotoxic, and factor Xa generation assays. While PRAN outperformed in the P-selectin exposure assay.

Conclusions

- PRAN is an inhibitor of thiol isomerase activity and P-selectin exposure. At high concentrations it is also effective in cancer cell destruction.
- Both PRAN and ZAF exhibit reversible inhibition properties.
- PRAN is inferior to ZAF in the inhibition of thiol isomerase activity, cancerous cell destruction and factor Xa generation.
- PRAN is more effective at cell receptor targeted assays than ZAF and could be due to PRAN's possible inability to enter cells, which would need to be further explored.

Discussion

Application of thiol isomerase inhibitors can improve patient outcomes greatly. Research has shown that the addition of a thiol isomerase inhibitor to a standard chemotherapy treatment can significantly reduce tumor growth and reduction of cancer cell induced thrombosis [5]. Furthermore, PRAN has shown to exude thiol isomerase inhibition properties that can directly relate to the inhibition of thrombus formation in cancer patients. Along with demonstrating reversibility which is important for an antithrombotic medication due to bleeding risks. However, direct cytotoxicity to cancer cells was minimal, which may be due to PRAN's possible inability or difficulty at entering the cell. PRAN may have certain clinical benefits in the area of cancer treatment and thrombosis. However, PRAN has been shown to be less effective than ZAF in multiple areas and further research pertaining PRAN may not be necessary.

References

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