**CXCR2 Small Molecule Antagonists: a novel approach to enhance the Effect of PD-L1 Inhibitors in Triple Negative Breast Cancer**

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1. **Background and Aim**

Immunologic checkpoint blockade (ICB) is a novel approach to reverse cancer immunosuppression and thereby promote immune responses against several cancers. Various studies observed the role of CXCR2 in tumour aggressiveness, resistance and immune-suppression (1-4). CXCR2 signaling is known to recruit myeloid derived suppressor cells (MDSCs) to the tumour microenvironment where MDSCs oppose the cytotoxic T-cell-mediated anti-tumor effect and suppress the efficacy of ICB. CXCR2 blockade significantly reduced the infiltration of MDSCs and improved the function of cytotoxic T cells.(5-6). CXCR2 inhibition was found to augment programmed cell death-1 (PD-1) inhibition in pancreatic cancer (7). CXCR2 inhibition has been proposed as an attractive anti-tumor treatment not only to enhance immunotherapy, but also to intensify the cytotoxicity of chemotherapeutic drugs. We have previously reported that the treatment of TNBC chemoresistant mammospheres with AZD5069 "a selective small-molecule antagonist of human CXCR2" sensitized the cells to Doxorubicin. Here, we investigate the potential use of AZD5069 in combination with Atezolizumab in an *ex-vivo* TNBC model.

2. **Materials and Methods**

(A) **Patient sample collection**

Breast cancer tissue biopsies and adjacent normal tissues were collected. Blood samples were collected from the same pool of patients where blood polymorph mononuclear cells (PBMCs) were isolated from TNBC patients’ whole blood and both tissues and PBMCs were preserved in -80°C.

(B) **RT-PCR Assay**

Total R.N.A was extracted from the tissue of breast cancer patients and their adjacent normal tissue. Quantification of the CXCR2 gene was done using qRT-PCR. Analysis was done using the 2−ΔΔCT method.

(C) **Ex vivo culture**

PBMCs were thawed gently then washed out by centrifugation, plated at equal density, stimulated by 1% (PHA) and incubated overnight in medium consisting of RPMI supplemented with 10% FBS and 1% pen/strep. After an overnight resting, the PBMCs were treated with AZD5069 doses. PBMCs were incubated for 48 hours after the treatment before being co-cultured with MDA-MB231 cells with ratio 10:1 respectively.

(D) **Lactate dehydrogenase (LDH) assay**

Cytotoxicity of Atezolizumab alone and in combination with AZD5069 in *ex-vivo* culture was detected using (LDH) assay kit. Cells were incubated for 72 hours after the drug treatment. Supernatant of each well was centrifuged and LDH reagents were used. LDH release was assessed by reading the absorbance at 490 nm.

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4a. **Results “CXCR2 Screening in Breast Cancer Tissue Biopsies”**

The expression level of CXCR2 is investigated using RT-PCR assay. CXCR2 was found to be highly expressed in the tumor tissue of breast cancer patients and is associated with worse clinical outcomes. Upon segregation of patients’ subtypes, TNBC and HER2 +ve patients showed a dramatic significant up regulation in CXCR2 expression compared to the controls (p = 0.0039) and (p= 0.0286) respectively. Hormonal Patients with ER +ve and/or PR +ve and HER2 –ve receptors showed mild significant up regulated CXCR2 expression (p= 0.0179). CXCR2 expression was significantly higher in TNBC patients compared to hormonal patients (p= 0.0199).

4b. **Results “LDH Cytotoxicity Assay of Atezolizumab and/or AZD5069”**

i) The combination of 30 nm AZD5069 (effective dose) and 200 nm Atezolizumab induced a significant additive effect in cytotoxicity than Atezolizumab alone (p = 0.0065)

ii) The non-effective dose of AZD5069 (10 nm) in association with Atezolizumab showed no increase in cytotoxicity than Atezolizumab alone (P = ns=0.0607)

5. **Conclusion**

Our data showed an additive effect of CXCR2 antagonists when combined with anti-PDL-1 immune check point inhibitor suggesting CXCR2 inhibition as a promising strategy to augment immunotherapy in TNBC.

6. **References**