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Research Article

Mechanism of Small Molecule Inhibitors of Phagocytosis

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Summary

Immune cytopenias occur when the body produces antibodies that target specific hematopoietic cells, inducing extravascular antibody-mediated phagocytosis by monocyte-macrophages in the spleen and/or liver through activation of Fcγ Receptors (FcγRs). Immune cytopenias include Immune Thrombocytopenia (ITP), Autoimmune Hemolytic Anemia (AIHA), Hemolytic Transfusion Reactions (HTR), Hemolytic Disease of the Fetus and Newborn (HDFN), and Autoimmune Neutropenia (AIN). Thus, novel therapeutics that inhibit phagocytosis would be useful, especially for short-term use while other therapies are being evaluated. In our earlier studies, we successfully identified two small-molecule drugs able to inhibit *in vitro* phagocytosis with a low IC_{50} concentration and negligible toxicity. These drugs, known as KB-151 and KB-208, have the potential to be utilized as lead compounds for further studies, once their mechanism of action is more clearly understood. In this regard, we have developed preliminary results that suggest that these small molecules may bind to the Fc receptors on monocyte macrophages and block the subsequent attachment of antibody-opsonized red blood cells to prevent phagocytosis.

Introduction

Individuals with immune cytopenias produce antibodies against certain hematopoietic cells in their blood [1-3]. Under these conditions, antibodies coat cells, allowing mononuclear phagocytes to recognize them through their surface Fc Receptors (FcR). Extravascular hemolysis occurs in the spleen and/or liver macrophages after recognition of the opsonized antibodies Fc region by the monocyte-macrophage FcRs, known as antibody-mediated phagocytosis [2]. The process can result in severe and potentially fatal complications for those who are affected [2]. The various types of immune cytopenias can be classified into six categories, which include autoimmune and alloimmune cytopenias [2]. Autoimmune cytopenias include: (i) Immune thrombocytopenia (ITP), which is characterized by specific autoantibodies to platelets, with increased platelet destruction in the spleen and liver, as well as decreased platelet production in the bone marrow; (ii) Autoimmune hemolytic anemia (AIHA), which involves the phagocytosis of autoantibody-coated red blood cells; and (iii) Autoimmune neutropenia (AIN), a rare disorder associated with autoantibodies produced against neutrophils that mainly affects children. Examples of alloimmune cytopenias include: (i) Hemolytic transfusion reaction (HTR), which occurs due to the phagocytosis of donor red blood cells as a result of preformed hemolytic alloantibodies to the donor red cell antigens; (ii) Delayed hemolytic transfusion reaction (DHTR), where the development of hemolytic alloantibodies occurs due to an anamnestic response to a transfusion due to a previous sensitization that is undetectable pre-transfusion; and (iii) Hemolytic disease of the fetus and newborn (HDFN), caused by maternal hemolytic IgG antibodies crossing the placenta into the baby's circulation and able to destroy baby's red blood cells.

Destruction via lysis within monocyte-macrophages of specific blood cells opsonized with antibodies is a hallmark of immune cytopenias and requires FcR-mediated phagocytosis. Nevertheless, the elucidation of novel mechanisms to mitigate phagocytosis would offer abeneficial therapeutic intervention [3-5]. Prior studiesfrom our laboratory have documented the chemical synthesis and assessment of multiple small molecules as potentially useful therapeutics for the treatment of ITP, and possibly other immune

cytopenias [6,7]. More recently, we have identified two small molecules that exhibit minimal toxicity and significant efficacy in inhibiting in vitro phagocytosis [5]. To obtain preliminary information regarding the mechanism of the inhibition, we examined the ability of these two molecules, KB-151 and KB-208, to inhibit FcR-mediated attachment of antibody-opsonized red blood cells.

Methods and results

Rosette assay

Using a variation of the established monocyte monolayer assay [8,9], PBMCs containing monocytes were allowed to adhere to chamber slides after 1 hr and 30 min at 37 °C with 5% CO_2 [8]. As previously reported, KB-151 and KB-208 were dissolved in 100% DMSO and adjusted to a final concentration of 100 µM in RPMI [5]. Following a one-hour incubation period, anti-RhD-opsonized red blood cells with Rh-positive antigens were introduced into the chamber slides. The slides were subsequently incubated for varying durations of 0, 5, 15, 30, 45, and 60 minutes at room temperature. Room temperature incubation allows for the antibody-opsonized red blood cells to attach to the monocytes FcRs but not be phagocytosed, forming so-called rosettes [10]. Following this, the samples were fixed and observed under phase-contrast microscopy to assess the formation of rosettes. Untreated cells were used as our control for rosette formation and as a positive control for inhibition, IVIG, known to inhibit phagocytosis due to the blocking of FcγRs, was used [5,8]. Inhibition of rosettes formation was started after 15 min and completed after 30 min (Figure 1).

The inhibition of phagocytosis after the rosettes formation was evaluated by adding 100 µM of KB-151 and KB-208 to the adhered monocytes at room temperature and incubating for 1 hour at 37 °C with 5% CO_2 (Figure 2). The number of phagocytosed RBCs in 300 monocytes was used to calculate the phagocytosis index (PI = (# of phagocytosed RBCs/300 monocytes) × 100) [5].

Discussion

There is a limited number of established treatments for severe manifestations of immune cytopenias. The majority of novel therapies have prioritized the treatment of ITP over other categories of immune cytopenia, including AIHA, HTR, (DHTR), and HDFN. Standard treatments for ITP and AIHA include corticosteroids such as dexamethasone and prednisone, as well as rituximab which is an anti-CD20 medication. Additionally, intravenous immunoglobulin (IVIG) and anti-D treatments are also utilized [11-13]. IVIG is considered second-line therapy for ITP; however, several options exist as alternatives to IVIG, such as thrombopoietin receptor agonists (TPOS-Ras), including Eltrombopag and Avatrombopag, anti-D, Spleen tyrosine kinase (Syk) inhibitors like fostamatinib, and splenectomy [14-18]. The efficacy of current therapies is limited by the time required for their effects to manifest. Consequently, patients with acute ITP, HTR, DHTR, HDFN, and fulminant AIHA, who are at risk of rapid cell destruction are also at risk for high morbidity and mortality. Hence, expeditious interventions that can counteract the immune-mediated eradication of particular

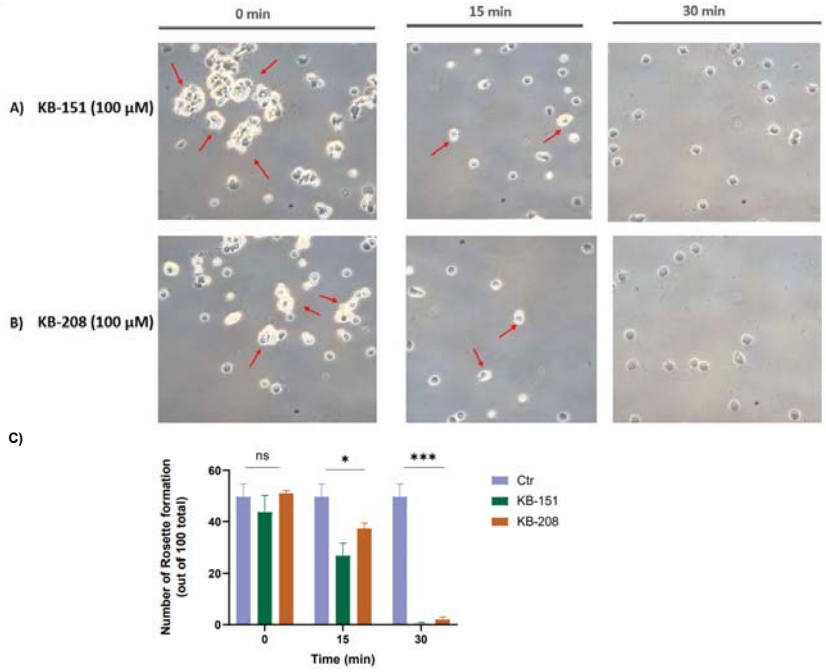

Figure 1: Rosettes formation under phase contrast microscopy- MMA was done at room temperature so as not to allow for phagocytosis which results in RBC rosettes forming; e.g., only attachment of anti-D-opsonized RBCs. (A) Human monocytes treated with KB-151 at 100 µM concentration for 0-, 15-, and 30-min. Rosettes formation (red arrow) started to be inhibited after 15 min and was completely inhibited after 30 min. (B) Human monocytes were treated with KB-208 at 100 µM concentration for 0-, 15-, and 30-min. Rosette formation started to be inhibited after 15 min and was completely inhibited after 30 min. (C) The number of rosettes formation. The rosette formation of each field is analyzed. Rosette inhibition began at the 15-minute mark and reached completion at the 30-minute mark. The sample size of *n* = 3 was utilized to count 100 monocytes in each chamber, which suggests the existence of rosettes.

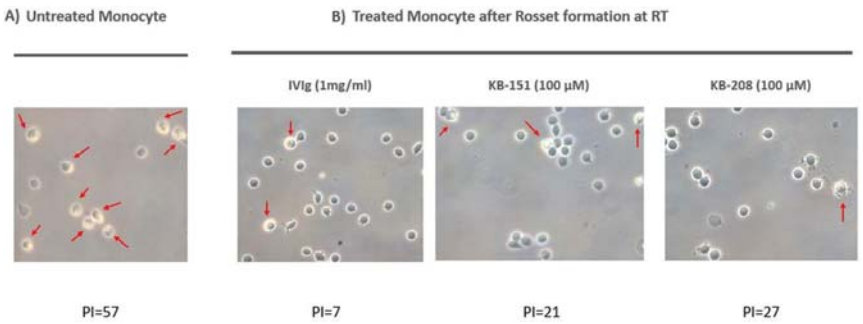

Figure 2: Phagocytosis after rosettes formation under phase contrast microscopy. MMA was done first at room temperature so that rosettes would form; e.g., only attachment of anti-D-opsonized RBCs. Then, the RT MMA was moved to 37 °C and optimal pH for phagocytosis to occur. (A) Untreated human monocytes representing monocytes phagocytosing RBCs opsonized with anti-D, after rosettes formation (see Figure 1), moved to 37 °C. (B) Human monocytes pre-treated for 15 min. with IVIg (1 mg/ml), KB-151 (100 µM), and KB-208 (100 µM) prior to moving to 37 °C- representing monocytes now phagocytosing RBCs opsonized with anti-D.

blood cells would confer significant benefits. More precisely, they would allocate supplementary time for the execution of alternative therapeutic approaches that would enhance the likelihood of the patient's improvement and survival. KB-151 and KB-208 significantly inhibited phagocytosis in human monocyte-macrophages at low µM IC_{50} concentrations with minimal to no toxicity *in vitro*, up to 250 µM, according to our previous work [5]. In the report herein, we provide preliminary findings that the inhibition of phagocytosis is due to the ability of the two drugs to inhibit the attachment of opsonized RBCs to FcγRs on the monocytes. In addition, the fact that these small molecules can work to inhibit phagocytosis when the rosetting RBCs at room temperature are moved to optimal conditions for phagocytosis (37 °C and buffered physiologic pH [8]), suggests that these small molecules are able to enter the cells to cause the phagocytosis inhibition. This may indicate that the mechanism of action of these small molecules may be to affect signal transduction pathways. To elucidate the precise mechanism of action, additional studies are required.

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