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Short communication

Identification of complement inhibitory activities of two chemotherapeutic agents using a high-throughput cell imaging-based screening assay

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ABSTRACT

Excessive complement activation contributes significantly to the pathogeneses of various diseases. Currently, significant developmental research efforts aim to identify complement inhibitors with therapeutic uses have led to the approval of one inhibitor for clinical use. However, most existing complement inhibitors are based on monoclonal antibodies, which have many drawbacks such as high costs and limited administration options. With this report, we establish an inexpensive, cell imaging-based high-throughput assay for the large-scale screening of potential small molecule complement inhibitors. Using this assay, we screened a library containing 3115 bioactive chemical compounds and identified cisplatin and pyridostatin as two new complement inhibitors in addition to nafamostat mesylate, a compound with known complement inhibitory activity. We further demonstrated that cisplatin and pyridostatin inhibit C5 convertases in the classical pathway of complement activation but have no effects on the alternative pathway of complement activation. In summary, this work has established a simple, large-scale, high-throughput assay for screening novel complement inhibitors and discovered previously unknown complement activation inhibitory activities for cisplatin and pyridostatin.

1. Introduction

Complement, a key component of innate immunity, serves as the first defense system against infection(Ricklin et al., 2010). Complement functions through one of three major activation pathways: classical, lectin, or alternative. The classical pathway is activated after antibody-antigen complexes bind with C1, the lectin pathway is initiated when certain exposed mannose or other sugar moieties bind to mannose-binding lectin (MBL) in serum. The alternative complement activation pathway is triggered by the spontaneous hydrolysis of the internal thioester bond of C3, subsequent binding of C3b to factor B, and activation of factor B by factor D. Following these initiation processes, the C3 convertases C4b2a for both the classical and lectin pathway, and C3bBb for the alternative pathway, form and subsequently cleave C3 into the small C3a and large C3b fragments. Subsequently, C3a, an anaphylatoxin, is released into the fluid phase while C3b is deposited on the cell surface at the complement activation site to facilitate phagocytosis and the formation of C5 convertase (C4b2a3b for the classical and lectin pathway, and C3bBb3b for the alternative pathway). C5 convertase then activates C5 to release C5a, another anaphylatoxin, as well as to generate the large C5b fragment, which initiates the assembly of membrane attack complexes (MAC, *a.k.a.*, C5b–9). The released anaphylatoxins promote inflammation by recruiting and activating leukocytes to killing the invading pathogens, additionally, the formed MACs cause osmotic cell damage or even lysis of the target cells.

However, activated complement must be tightly controlled to maintain homeostasis. Notably, the integral involvement of excessive complement activation has been identified in the pathogenesis of many human disorders, including cancer(Hajishengallis et al., 2017), age-related macular degeneration (AMD)(Edwards et al., 2005; Hageman et al., 2005; Klein et al., 2005), ischemia/reperfusion-induced injury (Danobeitia et al., 2014), and transplant rejection(de Cordoba et al., 2012). Consequently, intensive developmental research has focused on biologicals with complement activation inhibitory activities as novel therapeutic agents(Ricklin and Lambris, 2016). One such monoclonal antibody (mAb), eculizumab, which is an anti-complement component 5 (C5) mAb, has been approved for the treatment of paroxysmal nocturnal hemoglobinuria (PNH)(DeZern and Brodsky, 2015), atypical hemolytic uremic syndrome (aHUS)(Mache et al., 2009), and myasthenia gravis(Farmakidis et al., 2018) with an revenue of approximately $3 billion in 2017 alone. Other complement inhibitors, such as anti-C5a(Riedemann et al., 2017) and anti-MASP-2 mAbs, are being

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investigated in various stages of clinical trials for the treatment of various diseases including hidradenitis suppurativa(Kanni et al., 2018), IgA nephropathy, and thrombotic microangiopathy, with promising results(Ricklin et al., 2018).

As demonstrated above, most currently investigated complement inhibitors are mAbs, which are effective but also have some disadvantages such as a high cost (~$500,000/patient/year for eculizumab)(Coyle et al., 2014), limited routes of administration, and potential antigenicity. Accordingly, interest has increased in therapeutics based on novel small-molecule complement inhibitors, which feature many benefits, including relatively low costs and multiple administration routes, e.g., oral and subcutaneous. However, the requirement for robust large-scale, high-throughput screening assays for the identification of promising lead compounds represents the first major hurdle to the further modification, evaluation, and development of small-molecule complement inhibitors.

Current hemolysis-based complement assays represent certain challenges for high throughput screening. Time-consuming centrifugation and supernatant transfer for optical density analysis as well as associated cost increase may hinder successful workflow development and screening process. Imaging-based assays may be considered as an alternative high throughput-friendly approach. In this report, we describe a low-cost, cell imaging-based, high throughput screening system for the rapid and efficient identification of potential complement inhibitors. Using this system, we screened a small molecule library and identified three leading compounds; of these, only one had been previously identified as a complement inhibitor.

2. Methods and reagents

2.1. Chemical Compounds

The Collection of Biologically Active Molecules (Collection3115) was compiled from the LOPAC library (Sigma, St. Louis, MO, USA) and Bioactive Compound Library (Selleck Chemicals, Houston, TX, USA). A total of 3115 mechanistically annotated and partially redundant compounds were subjected to screening. All compounds were dissolved in DMSO to a stock concentration of 10 mM. The final DMSO concentration did not exceed 0.1% in the screening assay or during hit validation, and the negative and positive controls contained the same vehicle percentage. Upon hit identification, all compounds were retested as 10 mM stock solutions purchased from Selleck Chemicals.

2.2. Cell imaging-based high-throughput screening

To prepare antibody-sensitized sheep erythrocytes (E^{shA}) for the screening, 1 ml of sheep erythrocytes (Hemostat Laboratories, Dixon, CA, USA) were washed with GVB-E for twice and suspended with 8 ml of GVB-E. 10 µl of rabbit anti-sheep erythrocytes serum (MP Biomedicals, Santa Ana, CA, USA) was added. After incubation of 30 min in 37 °C and another 30 min on ice, the antibody-sensitized erythrocytes were washed and re-suspended. For high-throughput screening, 384-well plates (#3707; Corning, Corning, NY, USA) containing final test concentrations (10 µM) of each chemical were prepared using a Janus liquid handling platform (Perkin Elmer, Waltham, MA, USA) equipped with a 50-nL pin transfer tool (V&P Scientific, San Diego, CA, USA). A total of 20 µL of phosphate-buffered saline (PBS) supplemented with 0.15 mM Ca^{2+} and 1 mM Mg^{2+} (PBS + CaMg) were dispensed into each well of the plates using an automated dispenser (MultiFlo FX; Biotek, Winooski, VT, USA). Subsequently, 20 µL of 2.5% *normal human serum (NHS) in PBS + CaMg were* added to the 20 µL of PBS + CaMg buffer with small-molecule compounds from Collection3115 or with DMSO, followed by the addition of 10^5 E^{shA} in 10 µL of the PBS + CaMg buffer. After a 40-min incubation at ambient temperature (22 °C), 20 µL of 2.5% glutaraldehyde were added to the wells to stop the reaction and preserve cell morphology. In each plate, two columns (32 wells) containing vehicle only (i.e., without NHS) and two columns containing NHS only were included as positive and negative controls, respectively.

2.3. Automated cell imaging and analysis

An Operetta high-content imaging system with a 20X objective (PerkinElmer) was used for cell imaging. E^{shA} were imaged using the digital-phase contrast mode of the Operetta system at 3–6 hr after the addition of glutaraldehyde. Image analyses and calculations were performed using Acapella and Harmony 4.1 software packages (PerkinElmer). Intact E^{shA} were identified based on the cell size (from 6 to 45 μm^2, shape (roundness > 0.8), and phase-contrast signal intensity (> 185 units). Three non-consecutive fields per well were imaged, analyzed, and averaged; this corresponded to approximately 10^4 E^{shA} in control wells without NHS. The average number of intact E^{shA} per field were determined for each well, and the percent inhibition was calculated using the following equation:

((CMPD − CONTROL_NHS)/(CONTROL_no_NHS-CONTROL_NHS)) ×100, where CMPD is the number of intact E^{shA} per field in wells treated with compounds, CONTROL_no_NHS is the number in control wells without NHS, and CONTROL_NHS is the number in control wells with NHS.

Assay quality and screening plate data quality were determined based on Z' factor calculations performed as described(Zhang et al., 1999).

2.4. Classical and alternative complement pathway inhibition assay

Classical complement pathway activation was assessed using E^{shA} in Gelatin Veronal Buffer supplemented with Ca^{2+} and Mg^{2+} (GVB++; 10 mM Barbital, 145 mM NaCl, 0.1% Gelatin, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.3 ± 0.15; Boston BioProducts, Ashland, MA, USA) (Morgan, 2000). In brief, approximately 5×10^6 E^{shA} were incubated with serially diluted NHS (20% to 2.5%) and different titrations (0–50 µM) of cisplatin or pyridostatin in GVB++ at 37 °C for 10 min. For negative controls, 5 mM EDTA was added to the buffer to inhibit complement activation. For the alternative pathway, rabbit erythrocytes (E^{rabb}) and Gelatin Veronal Buffer supplemented with Mg^{2+} & EGTA (GVB-Mg-EGTA; 10 mM Barbital, 145 mM NaCl, 0.1% Gelatin, 0.5 mM $MgCl_2$, 10 mM EGTA, pH 7.4 ± 0.2; Boston BioProducts,) were used (Morgan, 2000). All the other conditions were the same as that in the classical pathway. After incubation, the erythocytes were centrifuged after the incubation, and the optical density (OD) at 414 nm (OD414) was measured in each 80 µl aliquots of supernatants containing released hemoglobin and used to calculate the percentage of hemolysis as follows: Hemolysis rate (%) = [(A − B)/(C − B)] × 100%, where A is the OD_{414} of tested samples, B is the OD414 of negative controls with EDTA, and C is the OD_{414} of maximum hemolysis induced by H_2O.

2.5. Classical complement pathway convertase assays

The effects of cisplatin and pyridostatin on the classical pathway C3 and C5 convertases were evaluated using E^{shA}, human C3 or C5-depleted serum (ComplementTech, Tyler, TX, USA), guinea pig serum (MP Biomedicals, Solon, OH, USA), and PBS + CaMg buffer according to a published protocol(Blom et al., 2014; Okroj et al., 2012). Briefly, E^{shA} were incubated at 37 °C for 5 min with 5% C3 or C5-depleted serum in 100 µl of GVB++ in the presence of various dosages of the compounds (0, 1.25, 2.5, 5,10 µM). In the absence of C3 or C5, C3 (C4b2a) or C5 convertase (C4b2a3b) assemble on the E^{shA} without further cascade initiation. After washing, 100 µl of 20 mM EDTA-GVB containing 3% guinea pig serum was added to each tube to initiate E^{shA} lysis via preassembled convertases. The samples were incubated for 20 min at 37 °C and centrifuged, and the OD414 of the supernatants were determined.