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Research Article

WIL2-S and an Engineered Jurkat Cell Line as a Model for Assessment of Apoptotic and Phagocytic Activity Upon Treatment with Anti-CD20

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Abstract

I-RmAb is an anti-CD20 monoclonal antibody, that we have developed as a therapeutic to treat B-cell malignancies. It induces cell death via various mechanisms such as complement dependent cytotoxicity (CDC), antibody dependent cell mediated cytotoxicity (ADCC), antibody dependent cell mediated phagocytosis (ADCP), apoptosis and sensitization of cancer cells to immunotherapy.

While other in-vitro assays are well-established, in this research, we focus on comparing Fab mediated apoptosis induced by I-RmAb in two CD20 positive human lymphoblast cell lines - WIL2-S and Raji, using flow cytometry, thus identifying a time window of peak apoptosis which has not been explored in depth before. This method is difficult to develop because I-RmAb elicits a very low apoptotic response and varies within different cell lines and patient derived cells such as B-CLL. We have also explored a luminescence and fluorescence method, and concluded that flow cytometry is a more reliable technique to assess apoptosis. Through this study, we hope to further narrow down the exact time window of apoptotic activity and a cell line sensitive enough to detect the same for developing a robust assay.

Alongside, we also outline a developed method that demonstrates I-RmAb's activation of the phagocytic pathway as a function of the FcγRIIa receptor in commercially available Jurkat cells harboring the NFAT reporter. Both apoptosis and ADCP assays are an important quality aspect for biosimilarity assessment with limited availability of experimental data in the literature. Hence, research under this wing will help improve the process of designing functional assays.

Keywords: Apoptosis; ADCP; Anti-CD20; WIL2-S

Abbreviations

PKC: Protein Kinase C; PLCγ: Phospholipase C γ; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; IP3: Inositol Triphosphate; ERK: Extracellular Signal-Regulated Kinase; MAPK: Mitogen-activated Protein Kinases; JNK: Jun N-terminal Kinase; DAG: Diacylglycerol; h: Hour; B-CLL: B-cell Chronic Lymphocytic Leukaemia; RLU: Relative Luminescence Units; RFU: Relative Fluorescence Units; PTK: Protein Tyrosine Kinase; CDC: Complement Dependent Cellular Cytotoxicity; ADCC: Antibody Dependent Cellular Cytotoxicity; ADCP: Antibody Dependent Cellular Phagocytosis; DRC: Dose Response Curve; ICH: The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; MOA: Mechanism of Action; RMP: Reference Medicinal Product

Introduction

CD20 is a B-cell antigen and a primary target for many B-cell malignancies. Monoclonal antibodies (mAbs) including biologics and biosimilars against CD20 antigen have shown selectivity and better clearance of tumour. Anti-CD20 mAbs can cross-link with

CD20 antigens on cell surfaces and induce killing of these cells via various mechanisms such as complement dependent cytotoxicity (CDC), antibody dependent cell mediated cytotoxicity (ADCC) and sensitization of cancer cells to chemotherapy. Two other suicide cascades that ensue post anti-CD20 treatment are Apoptosis and ADCP [1,8,12,15,22,24,25,32], which will be the focus of this manuscript.

Cross linking of CD20 receptor with anti-CD20 mAbs induces apoptosis [5,14,22]. Crosslinking of these mAbs has been shown to upregulate serine/threonine and protein tyrosine (PTK) kinases such as the Src family. Treatment with anti-CD20 alone or when hyper-crosslinked with a secondary antibody, mobilises calcium from the endoplasmic reticulum (ER), by subsequent activation of inositol triphosphate via phospholipase C γ [6,7,14,27]. Hyper-crosslinking of CD20 with secondary antibody such as goat anti-(mouse IgG) also enhances the level of apoptosis [26]. It has also been demonstrated that anti-CD20 apoptosis is mediated by caspase- 3 and caspase - 9 via cleavage of classic death substrate-poly(ADP-ribose) polymerase (PARP) [4,27]. Studies in the same paper [27] have evaluated the effect of Bcl-2 family proteins which are crucial regulators of apoptosis. They observed in Ramos cells that simultaneous expression of pro-apoptotic Bad protein and anti-apoptotic Bcl-2 protein may negate the effect of the latter due to its sequestration by Bad protein. The ratio of expression of these proteins did not change upon treatment with anti-CD20. Another study in SU-DHL-4 and BL60-2 cell lines showed upregulation of Bax protein (proapoptotic) [19]. In patient derived B-CLL (B-cell chronic lymphocytic leukaemia) cells, anti-apoptotic proteins such as XIAP and Mcl-1 were found to be downregulated post anti-CD20 treatment [4]. Anti-CD20 mAb also caused a strong and sustained phosphorylation of Mitogen-activated protein kinases (MAPKs) such as p38, extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK), where inhibition of MAPKs resulted in a significant decrease of apoptotic response [19,23]. A different mechanism of action for apoptosis was observed in a separate study, wherein, crosslinking CD20 on Ramos cells with anti-CD20 mAb caused changes in mitochondrial transmembrane potential, cytochrome c release and caspase activation independent of Bcl-2 levels [31]. A schematic representation outlining the mentioned events in the signaling cascade of apoptosis can be seen in figure 1A. Due to the complexity of apoptotic responses owing to different mechanism of actions (MOAs) and the expression profile of proteins in the signaling cascade, developing a reproducible and robust assay for apoptosis remains a challenge.

The mechanism of action (MOA) for ADCP relies on the ability of antibodies to act as a bridge between an infected/tumor cell and an immune cell, thereby promoting clearance of the infected/tumor cell [13]. The *in-vivo* binding of the Fab region of anti-CD20 mAb to the CD20 antigen on B cells, recruits monocytes, macrophages, neutrophils and dendritic cells, henceforth called effector cells [2,3,9,11,29,30]. These cells express FcγRIIa receptors on their surface that bind to the Fc-region of anti-CD20 mAb, rendering them metabolically active [9,12,13,28]. This leads to ingestion and degradation i.e. phagocytosis of the targeted B-cells by the effector cells, causing clearance of opsonized target cells which is known as Antibody Dependent Cell Phagocytosis (ADCP) [16,21,28]. A schematic of this MOA can be seen in Figure 1B. Despite the signaling events being straightforward, the development of an assay for ADCP was a challenge earlier, because primary cells were required to be isolated, differentiated into macrophages and validated, proving the need for a simpler, reproducible and time-saving method [5,13,16,18].

The importance of developing methods for such assays in the first place is to showcase and statistically prove functional similarity of biosimilars for regulatory submissions. We have developed an anti-CD20 biosimilar monoclonal antibody called I-RmAb, with a long term goal of marketing it worldwide. The development being in the pre-clinical stage, we would like to maintain confidentiality about the innovator molecule that this biosimilar is of. Although in-vitro assays for assessment of CDC and ADCC activity are well established, assays for apoptosis and ADCP are difficult to develop because of the reasons stated above including but not limited to variability in responses, expression profile of proteins and complexity of assay design. So far, apoptotic activity has been assessed in various CD20 expressing cell lines such as Raji, Daudi, Ramos, BL60-2, DHL-4 and patient derived cells such as B-CLL, with variable responses in different cells/cell lines [4,10,19,23,25]. However, not much has been explored in WIL2-S [20], a B lymphoblast cell line derived from a 5 year old male suffering from hereditary spherocytosis. Hence, in this research, we aimed to evaluate if WIL2-S can be a better model for development of apoptosis assay in comparison to

the commonly used Raji cell line. As part of our efforts for assay development, we demonstrate unexplored areas under apoptotic activity post anti-CD20 treatment and a simple method for ADCP.

Figure 1: Downstream effects induced by anti-CD20. Schematic representation of signaling events in apoptosis (A) and ADCP (B).

Materials and Methods

Cell lines and reagents

- **Cell lines:** Commercially available WIL2-S (B lymphoblast) and Raji (B lymphocyte) were used. Commercially engineered Jurkat T cells expressing FcɣRIIa (H variant) and harbouring the NFAT Reporter for luciferase gene were used for ADCP (referred as ADCP Effector cells in text). As a positive control in apoptosis experiments, Jurkat T cell line was used as a model cell line (referred as Jurkat cells in text).
- **Reagents:** Unless otherwise stated, reagents were procured from Sigma-Aldrich or Himedia. For maintenance of WIL2-S and Raji cell lines, RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, 1 mM sodium pyruvate, 1x antibiotic solution, 4.5 g/L glucose and 10 mM HEPES buffer was used. For the maintenance of the engineered Jurkat cell line, media supplemented with 200 µg/mL hygromycin B (Sigma-Aldrich) and 1000 µg/mL G418 (Sigma-Aldrich) was used. RMP of I-RmAb was commercially procured.

Apoptosis assay

Assessment of apoptotic activity was performed using flow cytometry. Annexin V has high affinity for phosphatidylserine (PS), a phospholipid that translocates to the extracellular region of the cell membrane during the early stages of apoptosis. During the late stages of apoptosis, the cell membrane loses integrity which can be tracked using propidium iodide (PI). Hence, Annexin V conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI) conjugated to phycoerythrin (PE) were used together (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences) for tracking both early and late apoptotic cell population.

Briefly, Raji and WIL2-S cells were harvested and seeded at a density of 0.3 x 10^6/well. To this RMP of I-RmAb was added at a final concentration of 0.05 µg/mL or 0.5 µg/mL. As a positive control, staurosporine (Sigma-Aldrich) at 2 µM concentration was added. Samples were incubated at different time points (5h, 16h, 20h, 24h, 48h) at 37°C with 85% humidity and 5% CO_2. Post incubation, cells were washed and stained using FITC Annexin V Apoptosis Detection Kit I as per manufacturers instructions (BD Biosciences). Cells were then acquired using BD FACSAria III and analysed using BD FACSDiva software. Cells that were Annexin V+/PI- were scored early apoptotic while Annexin V+/PI+ were scored late apoptotic/necrotic (Figure 2A). For luminescence/fluorescence plate based assay, Jurkat, Raji and WIL2-S cells were seeded at 10,000 cells/well. To this 2 µM staurosporine, a known

apoptosis inducer was added. Staurosporine treated Jurkat cell line which is a known model for apoptosis induction, served as a control. Kit (commercially procured, undisclosed) containing proteins and buffer for detection were added and the plate was incubated at 4h, 6h, 24h, 30h, 48h at 37°C with 85% humidity and 5% CO_2. Plate data was captured using a multimode reader (Molecular Devices) as per manufacturers instructions. Annexin V+/PI- cells were captured as relative luminescence units (RLU) and Annexin V+/PI+ cells were captured as relative fluorescence units (RFU) (Figure 2B).

Figure 2: Method overview. Apoptosis assay by Flow cytometry (A) and Luminescence/fluorescence capture (B).

ADCP assay

The ADCP assay that we developed is a luciferase reporter assay, in which the engagement and activation of the Fc receptor that is responsible for this process is measured and not the actual phagocytosis of any cells. In the present work, we demonstrated the mechanism using WIL2-S cells as target cells and engineered Jurkat cells [stably expressing FcγRIIa receptor and response element NFAT (Nuclear factor of activated T-cells driving expression of firefly luciferase)] as effector cells. WIL2-S and the effector cells were incubated with varying concentrations of I-RmAb or its RMP. D-luciferin, a non-luminescent, oxidized form of luciferase substrate was used to measure the activity. Firefly Luciferase, a class of oxidative enzyme, oxidizes ATP-dependent D-luciferin to oxyluciferin producing light emission. The bioluminescence emitted from this reaction depended on the number of luciferase enzyme molecules generated due to the activation of NFAT through cross linking of I-RmAb/RMP to FcγRIIa receptor of the effector cell, hence was directly proportional to the concentration of I-RmAb/RMP and its ADCP activity (Promega technical manual, G9901).

Briefly, WIL2-S cells were seeded at 0.5 x 10^6/mL in RPMI 1640 + 0.1% BSA media to which different concentrations of RMP and samples in the range of 5000 ng/mL -1.56 ng/mL were added. ADCP effector cells were added at 2.5 x 10^6/mL density and plates were incubated for 6h at 37°C with 85% humidity and 5% CO_2 incubator. After incubation, an equal volume of luciferase reagent (BPS Bioscience) was added to each well at room temperature. Plates were incubated in shaking condition on a shaker for 20 minutes and bioluminescence was captured using a multimode reader (Figure 3).

Figure 3: Method overview of ADCP assay.

Results and Discussion

WIL2-S showed maximal apoptotic activity at 16h

Apoptotic response to RMP of I-RmAb was evaluated at 5h, 16h, 20h, 24h and 48h in Raji and WIL2-S cell lines by flow cytometry (Figure 4A and 4B). 16h showed maximal apoptotic activity among other time points evaluated. Both Raji and WIL2-S cell lines showed a fairly good apoptotic response at 16h and 20h (15% and 30% respectively), with WIL2-S showing a slightly higher response than Raji for early apoptotic population. At 5h, 24h and 48h, Raji showed negligible response to the RMP of I-RmAb. At 24h, WIL2-S showed a fair response (15%), while at 5h and 48h, the response was negligible. Significant difference in apoptotic activity was observed between 0.05 µg/mL and 0.5 µg/mL in WIL2-S while the response was similar in Raji cell line for both concentrations.

WIL2-S, a less explored cell line showed a higher response (~30%) than Raji (~15%) at 16h. This is the first time that different time points have been explored for apoptotic activity in WIL2-S. The reason for such variable responses in WIL2-S and Raji could be that sensitivity and time window for apoptosis varies from cell line to cell line depending on the expression profile of CD20 and Bcl-2 family proteins (pro and anti-apoptotic). WIL2-S

may possess more CD20 antigens on the cell surface, more pro-apoptotic proteins and/or fewer anti-apoptotic Bcl-2 proteins than Raji which may explain the higher response in WIL2-S. Similar to research performed in other cell lines, evaluation of CD20 expression; calcium mobilisation; mRNA and phosphorylation profiles of p38, PTK and Bcl-2 in WIL2-S may help in correlating its apoptotic responses. This may also assist in developing and/or selecting a good model for apoptosis assay. We acknowledge that the data we present is preliminary in nature and that there is a requirement to perform more experiments as mentioned above, which stands in our future plan.

Figure 4: WIL2-S showed a better response at 16 h.

Comparison of early and late apoptotic responses to RMP of I-RmAb in Raji (A) and WIL2-S (B) cell lines at 5h, 16h, 20h, 24h and 48h.

Flow cytometry proved to be a better method for detecting apoptosis than plate based kit

To save time, achieve high throughput and avoid dependency on flow cytometry equipment, we tried to explore a commercially available kit that has the same principle of detection using Annexin V and DNA intercalating reagent using staurosporine as the apoptosis inducer. Data showed a trend in fluorescence activity

i.e. late apoptosis (Figure 5C), however luminescence data (early apoptosis) was inconclusive (Figure 5B). When the same experiment was analysed by flow cytometry, both early and late apoptotic activity as per the cell line profile was detected at the evaluated time points (Figure 5A). Even though cell densities varied, a trend for both fluorescence and luminescence activity was expected to be observed for kit based method. Plate based method indeed offered better flexibility and ease of data acquisition. However, flow cytometry was found to be a better method for detecting both early and late apoptosis. Since early apoptotic response is of prime interest, we found flow cytometry to be a better method for a reliable development of the assay. We acknowledge that there are other kit based methods available in the market such as detection of caspase activity which may be evaluated for simple and faster methods. But it has to be taken into consideration that caspase substrate is highly unstable with long term storage and multiple freeze thaw cycles, which may impact the reproducibility of data, as opposed to Annexin V and PI based reagents, which are much more stable.

Figure 5: Flow cytometry detected varying levels of apoptotic response in a distinguishable manner. Flow cytometry (A) detected both early and late apoptosis in all cell lines at 5h, 24h, 48h, however plate based kit (B, C) was able to detect late apoptosis alone (C).

ADCP assay was successfully established using an engineered cell line

In the development of ADCP assay, the concentration range was first evaluated using previously developed (in-house) ADCC assay as a reference. It was observed that concentrations ranging from 5000 ng/mL to 1.56 ng/mL achieved saturation at both ends of the DRC with a significant number of concentration points in between (Figure 6A). Once concentration range defining lower and upper asymptotes were identified, test accuracy was performed at 80%, 100% and 120% of RMP of I-RmAb with percentage recovery between 80-105% (Figure 6B). Finally, biosimilarity of I-RmAb was evaluated with RMP with a relative potency of 80-125% (Figure 6C). Functional similarity was successfully demonstrated with I-RmAb with a proper dose response curve (DRC). Differences in efficacy were observed, likely due to cell lines being at a high passage number.

Figure 6: Successful development of ADCP assay. (A) Concentration optimisation, (B) Test accuracy and (C) Functional similarity of I-RmAb to its RMP.

Conclusion

The purpose of this research was to highlight that WIL2-S, a less explored cell line, could be a better model than Raji to assess biosimilarity for apoptotic activity. More in depth study into the mechanism of action such as expression profile of CD20 antigens on B cell surfaces; mRNA and phosphorylation profiles of p38, PTK and Bcl-2; and assessment of calcium mobilisation may prove useful in better design of the assay. This may also aid in the development of therapeutics against B-cell malignancies. Since engineered cell lines reduce turnaround time significantly for the development of assays such as ADCP, it would be useful to consider the generation/usage of engineered cell lines for other such assays that require primary cells. As an immediate goal, the lab would aim to further complete development of the method in terms of method robustness and validation/qualification as per ICH guidelines for both apoptosis and ADCP assay. Preceding this paper, our lab presented this work at the Biosimilar Workshop 2023 conference in the form of a poster.

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