Efficacy of Different Immunological Approaches Targeting CD22 for the Treatment of Relapsed or Refractory Acute Lymphoblastic Leukemia: A Research Protocol

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Abstract

Introduction: Monoclonal antibodies (mAbs) have emerged as a promising immune-oncological approach to target cancer cells. mAbs have been seen to outperform traditional drug treatments in treating severe cancers despite their low relative cytotoxicity due to their high selectivity. CD22 is expressed in 60-90% of individuals with B-cell Acute Lymphoblastic Leukemia (B-ALL), and is rapidly internalized when bound to an antibody, making it an effective point of entry for cytotoxic agents. Epratuzumab is an anti-CD22 mAb, effective against B-ALL. Epratuzumab-SN-38 (Emab-SN-38) and Inotuzumab ozogamicin (InO) are promising anti-CD22 Antibody-Drug Conjugates (ADCs).

Methods: Epratuzumab, Inotuzumab, and Emab-SN38 treatments will be evaluated in vitro and in vivo. B lymphocytes collected from a 30-35-year-old R/R ALL patient will be purified and expanded. A cell culture assay will evaluate the treatments. Cells will be engrafted into humanized mice. Mice will be assorted into four treatment groups: saline (control), Epratuzumab, Inotuzumab, and Emab-SN-38. Quantitative flow cytometric analysis will be used to assess treatment effectiveness. Complete Response will be determined as ≅ zero human leukemic cells, Partial Response as ≤5% cells, and Remission as >5% cells or with identifiable clinical signs. Mice will be followed for 6 months after the last dose of treatment to assess for relapse and survival rate.

Results: It is expected that all three treatments will result in more significant results regarding tumour shrinkage and rate of cancer growth than saline. The ADCs are expected to perform better than unconjugated Epratuzumab. Relapse and Adverse Event rates are expected to be lowest in Epratuzumab-SN-38.

Discussion: The comparison of the effectiveness of these treatments are expected to establish Emab-SN-38 as a potential treatment option and propel research into other cytotoxic agents which could be used in conjugation with Epratuzumab and other mAbs.

Conclusion: ADCs combine the cytotoxicity of chemotherapy and the specificity of mAbs to treat R/R ALL. The ADCs are expected to outperform Epratuzumab in decreasing leukemic cell load given their potent targeted cytotoxicity. Emab-SN-38 is expected to be less toxic but as effective as Inotuzumab. These results could inform research on safer and more potent ADCs in treating R/R ALL via CD22.

Keywords: monoclonal antibody; antibody drug conjugate; Inotuzumab; Epratuzumab; cancer; leukemia; immunotherapy

As cancers continue to develop resistance to traditional drugs, a higher potency of cytotoxicity is needed, especially in the case of R/R ALL (Relapsed or Refractory Acute Lymphoblastic Leukemia). Off-target damage by increasingly potent chemotherapy often leads to the discontinuation of treatment due to worsened quality of life [1,2]. Given the severity of R/R B-ALL, immunological approaches have been investigated as a way to mitigate the adverse effects of chemotherapy and increase the survival rate of patients.
Monoclonal antibodies (mAbs) have emerged as a promising immune-oncological approach to targeting tumour cells, and dozens have been approved against cancers by the FDA, including R/R B-ALL [4,5]. MAbs consist of tumour-associated antigens targeting specific immune mechanisms within target cancer cells, and are thus able to effectively target and destroy them [2]. Mechanisms of mAbs include inhibiting tumour growth by blocking tumour angiogenesis and targeting checkpoint signals to improve the anti-cancer immune response [2,6]. Compared to traditional drug treatments, mAb agents may have greater potential in extending median patient survival duration and increasing remission [6]. Despite the aforementioned benefits of mAbs in its ability to efficiently target cancer cells, one major limitation of mAbs in comparison to traditional chemotherapy is its insufficient cytotoxicity against cancers and the inconsistence that occurs between mAb drug batches [5,7-9]. This low cytotoxicity requires high doses of the administration of mAb therapeutics to achieve clinical efficacy [10], leading to increased off-target effects and high drug production costs [6].

Antibody-drug conjugates (ADCs) are a novel approach for cancer immunotherapy involving the conjugation of a cytotoxic agent to a mAb to allow for more sustained and targeted therapy [11]. This addresses some of the aforementioned mAb limitations [11,12]. ADCs combine the specificity of mAbs with the potency of anti-tumour drugs to find cancer cells and deliver a strong cytotoxic payload [12,13]. Off-target toxicity is potentially minimized, improving the safety and efficacy of the treatment [13].

CD22 has recently been identified as a promising target for mAb-based therapy [14]. CD22 is a transmembrane glycoprotein present on mature B-cells and highly expressed on many types of malignant B-cells. It is essential for cell function, mediating survival and apoptosis [15,16]. CD22 is a target given that it is expressed in 60-90% of individuals with B-ALL [14]. In addition, CD22 has the ability to rapidly internalize when it binds to an antibody, making it an effective point of entry for cytotoxic agents [2,14].

Epratuzumab is a recombinant humanized mAb, originally derived from a mouse that underwent modifications to increase similarity to human antibodies. Epratuzumab binds with a great degree of specificity to CD22 and promotes antibody-dependent cellular cytotoxicity of cancer cells [17]. Anti-CD22 ADCs include Epratuzumab-SN-38 (Emab-SN-38) and Inotuzumab ozogamicin (InO).

Anti-CD22 ADCs include Epratuzumab-SN-38 (Emab-SN-38) and Inotuzumab ozogamicin (InO). Emab-SN-38 is the conjugation of the Epratuzumab mAb to a topoisomerase I inhibitor to enhance cell killing potential [2]. Emab-SN-38 has been demonstrated to be a promising minimally toxic ADC compared to other Epratuzumab ADCs. However, it has only been investigated once in a trial by Sharkey et al. [18].

Inotuzumab ozogamicin is a humanized anti-CD22 mAb conjugated to the cytotoxin calicheamicin [2]. Upon internalization, calicheamicin moves to the nucleus of the target cell to arrest cell division [19]. InO is a well-established anti-CD22 drug, showing high response rates in the treatment of R/R ALL in recent trials [20-22].

This article proposes a protocol to compare and evaluate the efficacy of these three immunotherapeutic approaches in the treatment of Relapsed or Refractory Acute Lymphoblastic Leukemia in humanized mice.

Methods

Cell Lines

The methods of this part of the procedure will be adapted from the protocol by DiJoseph et al. in their study of CD22-specific antibody-targeted chemotherapy using InO [23]. Human leukemic B cell lines will be derived from fresh human bone aspirates and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES (N-2-hydroxyethyl piperazine-N’-2-ethane sulfonic acid), 1 mM sodium pyruvate, 0.2% glucose, penicillin G sodium 100 U/ml, and streptomycin sulfate 100 μg/ml [23]. Before use, viable cells will be isolated by density-gradient centrifugation (30 min at 1000 g). The expression of B-lymphoid lineage-specific antigen CD22 on the surface of the B-ALL cell lines will be confirmed via flow cytometry [24].

The effect of the unconjugated Epratuzumab, InO, and Emab-SN-38 treatments on the growth of leukemic cells will be examined throughout 96 hours via in vitro culture assays [23]. Unconjugated CalichDMH will be used to assess the sensitivity of these cell lines to the calicheamicin toxin itself as a control group [23]. Effects on B-ALL cells or B-lymphoma will be assessed using a cellular variability indicator to determine the number of surviving cells following exposure to various drug treatments. All human leukemic B cells will be included in the cell line, rather than using solely CD22 cells. This allows for two assessments: firstly, the measurement of the CD22 cell killing potential of each treatment group (Emab-SN-38, InO, and unconjugated Epratuzumab) can be performed. Secondly, it can be determined whether each treatment group preferentially kills CD22 cells as opposed to other B cells, thus investigating off-target toxicity.

Cells will be seeded in a 96-well microtiter plate at a density of 5000-10,000 cells per well and exposed to various concentrations of the treatments [23].

Animal Model

For the following steps in experimentation, BRGSF-HIS mice will be used [25]. BRGSF-HIS mice contain all major human hematopoietic cell subsets, such as B cells, T cells, and NK cells, as described by their supplier genOway [25]. All experimentation will be conducted in accordance...
with the Canadian Council for Animal Care (CCAC), the appropriate institutional Animal Utilization Protocol (AUP) and following supplier recommendations [25,26]. Mice will be assessed for clinical signs of morbidity and will be euthanized as per the approved AUP.

24 mice will be used for the experiment with 6 mice per group. Sample size was decided in accordance to the number of mice needed to achieve statistical significance as per number of variables, outlined in the rough calculation of the “E” value [27]. The E value can be calculated by subtracting the total number of groups from the total number of animals.

Human Donor Cells

The protocol for the following two sections will be adapted from the protocol by DiJoseph et al. and Holmfeldt and Mullighan [23,28]. Leukemic human B cells will be collected from a 30-35 year-old R/R ALL patient [29] and isolated using the method described in the Cell Lines section. For injection, collected cells will be enriched using a density gradient centrifugation in a laminar flow biosafety cabinet soon after collection of the tissue [23].

Purification of primary B-ALL cells will be conducted via Fluorescence-Activated Cell Sorting (FACS) to avoid graft-host disease in mice [28,30]. This process involves tagging purified B-ALL cells (based on immunophenotype) with Alexa Fluor® 647 fluorescent antibodies, which are then detected through flow cytometry [31]. This tag will also be used to determine the level of engraftment of B-ALL cells [28].

Purified cells will be suspended in Matrigel (Collaborative Biomedical Products, Belford, MA, USA, diluted 1:1 in RPMI-1640 medium) [23]. Cells will be used immediately after collection. If immediate use is not possible, cells will be cryopreserved in liquid nitrogen and thawed immediately before use [28]. Human patient ALL specimens will be obtained through clinical tissue banks collecting tumour specimens with patient consent and confidentiality, in accordance with an appropriate Institutional Review Board.

Engraftment of Cells into Mice

5 × 10⁶ B lymphocytes will be engrafted into the mice. Mice will be put under a heating lamp to dilate their tail vein and the cells will be injected upon disinfecting the tail. Mice will be monitored closely for signs of host-graft disease, infections, and the development of leukemia. The mouse monitoring signs that will be used were outlined by Holmfelt and Mullighan [28]. Engraftment will be measured 4-5 weeks after transplant [28]. Peripheral blood will be obtained via blood draw from the tail vein and once 5% of human leukemic cells are seen in the blood measured using FACS, the experiment will begin [28]. If the threshold surpasses 50%, or according to the AUP and Institutional regulations, the mouse will be euthanized [28].

Experimental Method

Mice will be randomly assorted into four treatment groups: saline (control), Epratuzumab, InO, and Emab-SN-38. The dose administered in each treatment will be specific to the treatment based on available literature including the following protocols: Steinfeld et al. on Epratuzumab, DiJoseph et al. on InO, and Sharkey et al. on Emab-SN-38 [23,24,31]. The treatment will be applied to each group twice, 4 days apart over the duration of 4 weeks [24]. Mice will be followed for 6 months after the last dose of treatment has been administered, to assess for relapse and survival rates. Response rates (RR), complete response rates (CR), and adverse effects (AE) will be assessed after these 6 months [2].

Evaluation of Treatments

The following quantitative flow cytometric analysis technique will be adapted from de Vries et al. [24]. Detection of CD22 expression levels will be done using Alexa Fluor® 647 anti-mouse CD22 Antibody to evaluate cancer levels [31]. CD22 expression levels on B-ALL cells will be expressed as a CD22 ratio, defined as MFI (Mean Fluorescence Intensity) of B-ALL cells divided by the MFI of CD22-negative lymphocytes (T and NK cells) from each mouse specimen. To ensure purity of cells being injected into mice, Fluorescence-Activated Cell Sorting (FACS) will be performed. A second level of purification will be conducted using magnetic cell sorting (MACS). Magnets will be conjugated to the CD22 marker in B cells [32]. Quantitative flow cytometric analysis of absolute numbers of viable cells will be performed using a modified cytotoxicity assay [24]. 7-amino-actinomycin D (Alexis Corp, Lausen, Switzerland) or propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) will be used to exclude dead cells, in order to determine the quantitative count of viable cells. Additionally, Doublet discrimination will be performed to identify single cells from overlapping double cells. This will be done by excluding observations deviating from the standard forward scatter area (FSC-A) and height (FSC-H) correlation parameters in collected flow cytometry data.

A complete response to the treatment will be evaluated as zero or low vestigial amounts of human leukemic cells [34]. Partial response will be evaluated as less than 5% human leukemic cells [28,34]. Remission will include mice with 5% or more human leukemic cells, or with identifiable clinical symptoms in accordance with the CCAC guidelines [24,28,35]. This assessment will also be conducted after the end of the 6 month period after the last administered dose to evaluate remission. Survival rates of mice will also be considered in terms of AE.

Results

There has never been a direct comparison experiment conducted between Epratuzumab, InO, and Emab-SN-38 [2]. Epratuzumab is often administered in conjunction with
chemotherapy, and Emab-SN-38 has not been sufficiently represented in literature [18]. As such, expected results will be an estimation based on related studies and procedures as previously cited. However, given how recent the studies evaluated are, it is not possible to ascertain whether the following estimates will approximate actual results under the proposed protocol.

It is expected that all three treatments will result in more significant results regarding tumour shrinkage and rate of cancer growth than saline [18,23,24]. The ADCs are expected to outperform unconjugated Epratuzumab [2,18]. This performance is expected to be seen in terms of improved CR and RR in the InO and Emab-SN-38 treatment groups. Relapse and AE rates, including mortality, are also expected to be lower in the ADC groups compared to Epratuzumab. Epratuzumab has been linked to some AE and off-target toxicity when administered, suggested to be due to the need for high doses of the treatment to be effective [10,36].

Inotuzumab ozogamicin is very well established in literature as an efficient ADC for treating R/R ALL, showing high success in recent trials by significantly improving CR, RR, and decreasing relapse rates [20-22]. However, it has been associated with severe adverse side effects such as veno-occlusive disease, liver toxicity, and generalized toxicity [37].

Emab-SN-38 was reported by Sharkey et al. to be potent against CD22 and highly effective against R/R ALL in mice well below toxic levels [18]. As such, it is possible to expect that Emab-SN-38 will present less severe side effects than InO, thus increasing its strength as a therapeutic approach. This is a crucial point to investigate in this proposed protocol given that this consideration has not been observed in literature.

As such, Epratuzumab-SN-38 is expected to outperform the Epratuzumab and Inotuzumab ozogamicin groups, leading to increased Complete Response rate, Response Rate, and decreased Adverse Effects.

Discussion
The aim of this study is to compare Epratuzumab, Inotuzumab ozogamicin, and Epratuzumab-SN-38 as immunotherapeutic approaches in their ability to treat R/R ALL in human leukemic cell lines and in humanized leukemic mice. The findings of this study are expected to better inform immunological based approaches towards cancer therapy and support the role of ADCs as targeted anti-tumour mechanisms of low off-site toxicity in comparison to conventional therapies [11-13]. The effectiveness of these treatments will be measured using cell viability indicators and antibody-based imaging techniques to determine the decrease in relative leukemic cell count [28,30,36]. The comparison of the effectiveness of these treatments is expected to establish Emab-SN-38 as a potential treatment option and propel research into other cytotoxic agents which could be used in conjugation with Epratuzumab and other mAbs.

Following the mice six months after the treatment allows for insight into the relapse rates of R/R ALL leukemia under the consideration that increased mortality is associated with prevalent relapsed leukemia [1]. Successful treatment observed in terms of CR could support the notion that immunological therapies such as Emab-SN-38 have the potential to be highly cytotoxic at low doses. With a more potent and targeted approach, these ADCs are efficient at minimizing off-target toxicity and may be optimized to use even stronger agents [11,12].

Future investigations may involve the combination of multiple ADC therapies, as well as in combination with unconjugated mAbs and traditional chemotherapy. It is important to evaluate the impacts of these combination therapies given their relevance in clinical settings, as well as what synergistic factors make combined treatments more effective.

A clear direction would be to transition from mice models to clinical trials. The use of mice for models allows investigators to improve the internal validity of the study at the expense of the external validity of the findings. The study design does not take into consideration many human factors and covariates which may affect the viability of the treatment plan. When informing treatment options, it is important to consider the quality of life and adherence especially in the case of the high AE rate of these treatments, something that cannot be tested on mice. Clinical trials involving ADCs or mAbs showed inconsistent results in varying human populations, including age as an example [2,36,37,39]. Thus, future research should attempt to consider age, sex, disease history, and other covariates which could potentially impact the strength of antibody-based therapies [40].

Conclusions
This study proposes a protocol to compare the efficacy of three monoclonal antibody-based techniques for the treatment of R/R B cell leukemia. The proposed protocol includes one unconjugated mAb, Epratuzumab, and two ADCs, Inotuzumab ozogamicin and Epratuzumab-SN-38. The ADCs are expected to outperform Epratuzumab in decreasing leukemic cell load, decreasing relapse rates, and increasing remission when targeting CD22 due to their increased cytotoxicity and target specificity [2,41]. Epratuzumab-SN-38 is a potentially less toxic alternative to Inotuzumab ozogamicin, and if determined to be more effective, will inform safer and more effective potent treatments for R/R ALL [18].

Potential for future research would include transitioning from in vivo to clinical trials to study the implications of each treatment method and observe long-term impacts on the prognosis of R/R ALL patients. SN-38 and calicheamicin as toxic agents could also be studied as future targets for conjunction with antibodies other than...
Epratuzumab. Studying the viability and impact of these cytotoxic agents on tissues and cancer cells could push the development of ADCs as a targeted and precise cancer therapy.

Overall, targeted immunotherapies including monoclonal antibodies, Antibody-Drug Conjugates, and combination therapies are promising approaches to treat cancers with poor prognosis, including Relapsed or Refractory Acute Lymphoblastic Leukemia.

List of Abbreviations Used
ADCs: antibody drug conjugates
AE: adverse effects
ALL: acute lymphoblastic leukemia
B-ALL: B cell acute lymphoblastic leukemia
CalichDMH: calicheamicin dimethyl hydrazide
CCAC: Canadian council on animal care
CD22: cluster of differentiation 22
CR: complete response rate
FACS: fluorescence-activated cell sorting
FDA: the food and drug administration
HEPES: N-2-hydroxyethyl pipperazine-N’-2-ethane sulfonic acid
mAbs: monoclonal antibodies
MFI: mean fluorescence intensity
RR: response rate
R/R ALL: relapsed or refractory acute lymphoblastic leukemia
RPMI-1640 growth medium: Roswell Park memorial institute-1640 growth medium
SN-38: 7-ethyl-10-hydroxycamptothecin

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent
All experimentation will be conducted in accordance with the Canadian Council for Animal Care (CCAC), and following recommendations by genOway. Primary patient ALL specimens will be obtained through clinical tissue banks collecting tumour specimens with patient consent and confidentiality, in accordance with an appropriate Institutional Ethics Review Board.

Authors’ Contributions
VMO: Contributed to the design, planning, and methods of the study, reviewed background information, collected and interpreted data, drafted and revised the manuscript, and gave final approval of the version to be published.
CJX: Contributed to the background information and methods of the study, as well as the interpretation of data and planning and methods, revised the manuscript critically, and gave final approval of the version to be published.
EZ: Contributed to the background and design of the study, as well as the interpretation of data and planning and methods, revised the manuscript critically, and gave final approval of the version to be published.

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