CD20-TCB with obinutuzumab pretreatment as next generation treatment of hematological malignancies

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Translational Relevance

Redirecting T cells to hematological malignancies with bispecific antibodies (TCBs) is an attractive strategy to improve outcome of patients with recurrent and/or refractory disease. However, such approaches frequently lead to severe side effects upon infusion requiring the introduction of step-up-dosing (SUD) regimens and steroid pre-treatment to enhance safety.

We developed a potent, next generation, half-life extended CD20-targeting TCB antibody (CD20-TCB), based on the 2:1 molecular format, which has significantly superior potency compared to other CD20-targeting TCBs in clinical development. In addition, we present a novel approach enabling safer administration of such a potent drug consisting of a single administration of obinutuzumab (Gazyva pre-treatment, Gpt) prior to the first infusion of CD20-TCB. Our data provide evidence that Gpt abrogated cytokine release of the first CD20-TCB administration more efficiently than SUD regimen, thus representing an attractive, safer and clinically-relevant approach for treatment of patients with hematological malignancies.
Abstract

Purpose: Despite promising clinical activity, T cell engaging therapies including T cell bispecific antibodies (TCBs) are associated with severe side effects requiring the use of step-up-dosing (SUD) regimens to mitigate safety. Here, we present a next generation CD20-targeting TCB (CD20-TCB) with significantly higher potency and a novel approach enabling safer administration of such potent drug.

Experimental design: We developed CD20-TCB based on the 2:1 TCB molecular format and characterized its activity pre-clinically. We also applied a single administration of obinutuzumab (Gazyva pre-treatment, Gpt) prior to the first infusion of CD20-TCB as a way to safely administer such a potent drug.

Results: CD20-TCB is associated with a long half-life and high potency enabled by high-avidity bivalent binding to CD20 and head-to-tail orientation of B and T cell binding domains in a 2:1 molecular format. CD20-TCB displays considerably higher potency than other CD20-TCB antibodies in clinical development and is efficacious on tumor cells expressing low levels of CD20. CD20-TCB also displays potent activity in primary tumor samples with low effector:target ratios. In vivo, CD20-TCB regresses established tumors of aggressive lymphoma models. Gpt enables profound B cell depletion in peripheral blood and secondary lymphoid organs and reduces T cell activation and cytokine release in the peripheral blood thus increasing the safety of CD20-TCB administration. Gpt is more efficacious and safer than SUD.

Conclusions: CD20-TCB and Gpt represent a potent and safer approach for treatment of lymphoma patients and are currently being evaluated in Phase I, multicenter study in patients with relapsed/refractory NHL (NCT03075696).
Introduction

The introduction of rituximab over 20 years ago marked the beginning of a new era in the targeted treatment of B cell non-Hodgkin lymphomas (NHL). Its combination with chemotherapy (R-CHOP) remains a standard of care for follicular NHL (fNHL), diffuse large B cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL), (1), but, prognosis is poor in patients who fail to respond to first-line treatment or develop resistance. Multiple attempts have been made to improve on anti-B cell therapeutic antibody therapy, including targeting alternative CD20 epitopes and alternative B cell surface targets (e.g., CD19 and CD22), modification of the Fc region, and development of antibody-drug conjugates. To date, only obinutuzumab (Gazyva), a glycoengineered Type II CD20 humanized antibody (2), has shown an advantage over rituximab in Ph III chemo-immunotherapy trials, demonstrating superior efficacy in first-line CLL and fNHL (3, 4), but failing to improve progression-free survival versus rituximab in first-line DLBCL (5). Despite these efforts, and parallel development of small-molecule-based targeted therapies (6-9), a significant unmet need remains for improved therapeutic options in NHL (10).

A more recent therapeutic approach involves redirecting T cells to attack B cells, either using bispecific antibodies that bind to a B cell surface target and common surface component of the T cell receptor (TCR) (e.g., CD3ε) (11, 12), or chimeric antigen receptor-modified T cells (CAR-T cells) that are genetically modified to express the target-binding domain of an anti-B cell antibody fused to a TCR intracellular signaling domain (13, 14). Blinatumomab, a T cell bispecific (TCB) antibody targeting CD19 and CD3ε is approved in relapsed/refractory B cell acute lymphoblastic leukemia (B-ALL; ref 15), and in clinical trials for NHL. Two CAR-T cell
therapies targeting CD19 are also approved: tisagenlecleucel-T for relapsed/refractory B-ALL (16), and axicabtagene ciloleucel for relapsed/refractory large B cell lymphoma after ≥2 lines of systemic therapy (17). While T cell re-directed approaches are promising as their mode of action is potentially complementary to classical IgG1 antibodies, their use in the broader NHL population may be limited. Blinatumomab, for example, must be administered by continuous infusion due to its short half-life. Further, step-up dosing (SUD) is required to mitigate side effects associated with initial TCB antibody infusions. Even with SUD, infusion-related reactions and CNS toxicity is still an issue for blinatumomab in DLBCL (12). CAR-T cell administration involves a 3-week turnaround time before administration – challenging in an aggressive disease setting – and specialized centers are required for its administration and management of associated neurological side effects/severe infusion reactions. Several IgG-based anti-CD20-directed TBC antibodies, all of which are monovalent for CD20, are in early clinical development (18-20). While these agents have the potential to overcome the frequent dosing limitations seen with blinatumomab, the need for SUD remains and their comparative efficacy with blinatumomab and CAR-T cells has yet to be determined.

Here, we present a novel CD20-targeting TCB (CD20-TCB; RG6026), which has a long half-life and high potency enabled by high-avidity bivalent binding to CD20 and head-to-tail orientation of B and T cell binding domains in a previously characterized 2:1 TCB molecular format (21, 22). Our preclinical investigations indicate that a single administration of obinutuzumab prior to the first infusion of CD20-TCB (Gazyva pre-treatment, Gpt) may represent a more robust approach to safely administer such potent drugs.
Materials and methods

**Cell culture for in vitro assays**

CCRF SB, Pfeiffer, SU-DHL-2, SU-DHL-5, Toledo cells (all American Type Culture Collection [ATCC]) as well as Jurkat, DoHH-2, OCI-Ly18, U2932 and WSU DLCL2 cells (all Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ]) and Z138 cells (gifted from the University of Leicester, Leicester, United Kingdom) were cultivated in RPMI 1640 medium (Gibco/Lubioscience # 42401-042) containing 10% fetal calf serum (FCS, Gibco) and 1% Glutamax (Invitrogen/Gibco # 35050-038). RC-K8 and SU-DHL-6 cells (both DSMZ) were cultivated in RPMI 1640 medium containing 15% FCS and 1% Glutamax. SU-DHL-8 and ULA cells (both DSMZ) were cultivated in RPMI 1640 medium containing 20% FCS and 1% Glutamax. SU-DHL-4 cells (DSMZ) were cultivated in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen/Gibco #42430-082) containing 10% FCS and 1% Glutamax.

Cell lines provided by ATCC and DSMZ are routinely authenticated by short tandem repeat profiling prior to delivery. Upon receipt, cells were expanded and frozen; cells were not passaged for more than 6 months after resuscitation. No further authentication of these cell lines was conducted. The Z138 cells were not authenticated.

**Binding to CD20- and CD3-expressing cells**

Median fluorescence intensities (MFI) were calculated for binding of CD20-TCB to B cells and primary CD4+ and CD8+ T cells freshly isolated from human and cynomolgus monkey peripheral blood mononuclear cells (PBMCs). Binding of CD20-
TCB to human Jurkat T cells, cynomolgus monkey HSC-F T cells, and human mantle cell lymphoma tumor line Z-138, was measured by flow cytometry as described in the Supplementary methods.

**Tumor cell lysis in vitro**

B cell-depleted PBMCs derived from healthy donors were prepared using standard density-gradient isolation followed by B cell depletion with CD20 Microbeads (Miltenyi Biotec) and incubated with CD20-TCB or untargeted-TCB, tumor targets (Toledo, U2932 or Z-138) at ratio of 5:1 for 20–24 h. The reason of using B cell-depleted PBMCs as effector cells was to enable an exclusive assessment and quantification of tumor target cell lysis, without the additional signal coming from the lysis of normal B cells (present within PBMCs), which are expressing CD20 and thus represent an additional target for CD20-TCB. Samples were incubated at 37°C and 5% CO₂ in a humidified incubator for indicated time points (all in triplicate). Lactate dehydrogenase (LDH) release was measured using the LDH Cytotoxicity Detection Kit (Roche Applied Science, Basel, Switzerland). Antibody-dependent-cellular cytotoxicity (ADCC) was calculated as follows

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\text{Percentage ADCC} = \left( \frac{\text{sample release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \right) \times 100.
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Spontaneous release, corresponding to target cells incubated with effector cells without TCB, was defined as 0% cytotoxicity and maximal release (target cells lysed with 2% Triton X-100) defined as 100% cytotoxicity. The average percentage of ADCC and standard deviations of the triplicates of each experiment were calculated. Flow cytometry was performed on PBMCs collected within 1-3 days of tumor cell lysis stained with FACS antibodies to show the *in vitro* kinetics of expression of T cell
activation (CD25, 4-1BB, Ox40, ICOS) and exhaustion markers (PD-1, Lag3). Supernatants derived from 20–24 h incubated tumor cell lysis assays with Z-138 and isolated pan T cells (Miltenyi Biotec) (effector:target 3:1) were analyzed by Cytometric Bead Array (CBA, BD Biosciences) to quantify cytokine release. T cell proliferation and endothelial adhesion/activation in vitro were also assessed (see Supplemental Methods for further details).

**Live in vitro imaging**

WSU-DLCL2 cells were plated overnight on Retronectin-treated (2 hrs at RT using 5 µg/cm²) 8-well Glass Bottom slides (Ibidi #07/2019) at 37°C to adhere and stained with CMAC dye (Molecular Probes, Life Technologies). Before acquisition, CD3/CD28-activated CD8-positive T cells were stained with CMTMR (Molecular Probes, Life Technologies) and plated together with tumor cells. Alexa647-labeled CD20-TCB (500 ng/mL) was added directly into the growth media. Slides were imaged with a confocal microscope (inverted LSM 700, Zeiss) with a temperature and CO₂ controlled stage. Live acquisition was performed with a 20x objective. Movies were collected using Zen software (Zeiss) coupled to the microscope, analyzed with Imaris (Bitplane; Oxford Instruments), and plotted using GraphPad Prism.

**Ex vivo assays using primary tumor samples**

*Ex vivo* assays were performed by Vivia Biotech (Madrid, Spain) incubating fresh and frozen bone marrow samples from adult leukemia and lymphoma patients with selected TCBs. Studies were conducted by Vivia Biotech in accordance with recognized ethical guidelines. T cells and target cells were analyzed by flow cytometry, target cell death determined by Annexin V.
**In vivo studies in mice and cynomolgus monkeys**

The routine care of all animals used in this study is described in detail in the Supplementary methods. Mice were maintained under specific-pathogen-free condition with daily cycles of 12 hours light /12 hours darkness according to international (Federation of European Laboratory Animal Science Associations) and national (Gesellschaft für Versuchstierkunde / Society of Laboratory Animal Science [GV-Solas] and Tierschutzgesetz [TierSchG]) guidelines. The study protocol was reviewed and approved by local government (license ZH193/2014).

CD20-expressing WSU-DLCL2 or OCI-LY18 cells were cultured at 37°C in a water-saturated atmosphere at 5% CO\textsubscript{2}. Cells were harvested, washed once with RPMI, re-suspended in RPMI and injected into female HSC-NSG mice SC at 0.1 mL/mouse (1.5 x 10\textsuperscript{6} WSU-DLCL2 or 10 x 10\textsuperscript{6} OCI-LY18 cells in 50% Growth Factor Reduced Matrigel admixed with 50% RPMI). CD20-TCB treatment started when tumor volume reached 100–150 mm\textsuperscript{3}, and was administered intravenously (IV) once weekly at the doses described later in the text. In some studies, mice received obinutuzumab pre-treatment (Gpt) at the dose of 10 mg/kg IV, one week prior to CD20-TCB administration. Antibody dilutions were freshly prepared prior to use.

Purpose-bred, naïve, Cynomolgus monkey (Macaca fascicularis) were obtained from Charles River Laboratories, Houston, TX (Origin: Mauritius). Animal were 3.5 to 4.7 years old and weighed 3.5 to 6.1 kg at the time of dosing. Animals were housed in stainless-steel cages. Primary enclosures were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the most current Guide for the Care and Use of Laboratory Animals (National Research Council. Guide for

Cynomolgus monkeys were treated with 50 mg/kg IV obinutuzumab to deplete B cells, 0.1 mg/kg CD20-TCB IV or vehicle control. IFNg in plasma samples were measured 4 h after treatment by bead-based sandwich immunoassay measured on a Luminex platform. LLOQ was 75 pg/mL.
Results

**Generation and characterization of CD20-TCB biological activity**

Three different TCB structural variants were generated and their biological activity compared (Figure 1A; supplemental Table 1). The first, 2:1 CD20-TCB, based on the 2:1 TCB format (21, 22), had two anti-CD20 Fabs and one anti-CD3 epsilon subunit (CD3e) Fab with one of the CD20 Fabs fused directly in a ‘head-to-tail’ fashion to the anti-CD3e Fab via a flexible linker. A heterodimeric human IgG1 Fc region carrying the ‘PG LALA’ mutations (23) was incorporated to abolish binding to Fcγ receptors and to complement component C1q whilst maintaining neonatal Fc receptor (FcRn) binding, enabling a long circulatory half-life. Two additional TCBs were based on 1:1 molecular formats (i.e., having one anti-CD20 Fab and one anti-CD3e Fab) named the 1:1 IgG CD20-TCB, an IgG-like molecule with each Fab fused to either N-terminus of the IgG1 hinge region, and 1:1 OA CD20-TCB, a one-arm (OA) molecule with both Fabs fused to one terminus of the hinge region in a head-to-tail fashion. The Type II anti-CD20, humanized Fab region was from obinutuzumab. Both anti-CD20 and anti-CD3e Fabs recognized their respective targets with similar affinity between human and cynomolgus monkey cells, e.g. binding EC_{50} of 4.8 vs. 3.3 nM, for primary human vs. cynomologus monkey B cells, respectively (supplemental Figure 1).

The superior biological activity of the 2:1 molecular format over both 1:1 TCB formats was demonstrated in tumor lysis experiments using target cell lines expressing various levels of CD20 antigen (supplemental Table 2) and healthy donor PBMCs derived from several individuals. 2:1 CD20-TCB displayed on average a 40 fold stronger potency (range between 12 and 95 fold) than the 1:1 CD20-TCBs (Figure...
1A; supplemental Figure 2A; supplemental Table 2) supporting the relevance of CD20 avidity/bivalency. Notably, the 1:1 head-to-tail format was more potent than the classical 1:1 IgG format, supporting the benefit of bringing both Fabs in close spatial proximity via a flexible linker compared with separating them on either side of an IgG1 hinge region.

The activity of 2:1 CD20-TCB was also compared with a CD20-targeting 1:1 TCB antibody with alternative anti-CD20, anti-CD3, and Fc sequences (derived from a TCB currently in Phase I trials), (19), and showed on average a 40 fold superior potency (range between 4-153 fold), (Figure 1B; supplemental Figure 2B; supplemental Table 2).

2:1 CD20-TCB also displayed strong activity ex vivo on primary tumor samples derived from aggressive lymphoma and leukemia patients (Figure 1C&D; supplemental Table 3). Even under low effector to target (E:T) cell ratios (0.02–0.8), and using only the existing T cells present in the primary samples, 2:1 CD20-TCB displayed high potency as illustrated by the low EC$_{50}$ values of target cell killing (Figure 1C) and profound target cell depletion (Figure 1D). Flow cytometric analyses of patient samples revealed efficient target cell depletion occurring as early as 24 h of incubation, which continued at subsequent time points (supplemental Figure 3).

Together, the 2:1 CD20-TCB displayed superior potency compared with CD20-TCB antibodies based on the classical 1:1 IgG format and was therefore selected for further development. The development molecule is referred as CD20-TCB in the remainder of the manuscript.
**Insights into the mode of action of CD20-TCB**

Live *in vitro* imaging of co-cultures of CD20-expressing lymphoma cells (WSU-DLCL2), pre-activated CD8 cytotoxic T cells, and fluorescently-labeled CD20-TCB enabled visualization of antibody binding and tumor cell lysis. Incubation with CD20-TCB notably reduced the speed of T cell movement and increased tumor/T cell synapse area, reflecting prolonged and larger interactions between tumor cells and T cells that correlated with CD20-TCB localization at synaptic areas (Figure 2A&B; supplemental Figure 4A; Video 1&2). Tumor lysis was observed already at 4 h with multiple T cells contributing to killing of a single target cell (Video 3). Flow cytometric analysis of samples collected at different time points confirmed T cell activation and expansion associated with tumor killing as evidenced by the expression of a broad panel of T cell activation and exhaustion markers (Figure 2C; supplemental Figure 4B), cytotoxic granule and cytokine secretion, and proliferation (supplemental Figure 5A&B). Both CD4 and CD8 T cell subsets expanded following tumor killing, but CD8 T cells underwent stronger proliferation as shown by higher number of proliferation cycles compared to CD4 T cells (supplemental Figure 5B).

**In vivo activity of CD20-TCB**

*In vivo* pharmacology studies performed in HSC-NSG mice (Figure 3A), showed that peripheral blood B cells were largely depleted within 24 h of the first CD20-TCB administration (0.5 mg/kg, once weekly IV) and remained undetectable for the rest of the study (Figure 3B). Pharmacokinetic (PK) data showed target-mediated clearance by B cell binding during the first TCB administration and IgG-like TCB PK for a subsequent administration once peripheral B cells were depleted (Figure 3C). Peripheral blood T cells decreased transiently within 24 h of the first CD20-TCB
administration but returned to baseline levels within 72 h and remained stable for the rest of the study (Figure 3B). The second and subsequent administrations of CD20-TCB did not induce such T cell decrease in the peripheral blood.

Flow cytometry showed that, 72 h after the first CD20-TCB administration, peripheral blood T cells were activated and both CD4+ and CD8+ T cell subsets were proliferating as demonstrated by the up-regulation of CD25, 4-1BB, PD-1, Granzyme B, and Ki67 (all hallmarks of T cell activation through TCR complex crosslinking; Figure 3D). Initial T cell activation in peripheral blood, during CD20-TCB-mediated B cell depletion, was further exemplified by transient cytokine release (IFNγ, TNFα, MCP-1, MIP-1β, IL-2, IL-5, IL-6, IL-8, IL-10) in blood of treated mice (measured at 24 h after the first CD20-TCB injection and normalizing to nearly baseline levels by 72 h; Figure. 3E; supplemental Table 4). Importantly, cytokine levels did not increase after a second administration of CD20-TCB (d10), consistent with the almost complete B cell depletion in the peripheral blood upon the first CD20-TCB administration (Figure 3B&E; supplemental Table 4), as CD20 binding is a pre-requisite for CD3 crosslinking on T cells by CD20-TCB. In addition to peripheral blood, CD20-TCB also efficiently eliminated B cells in the spleen and lymph nodes (Figure 3F).

The in vivo anti-tumor activity of CD20-TCB was assessed in HSC-NSG mice bearing an aggressive DLBCL xenograft model (WSU-DLCL2), poorly responsive to anti-CD20 IgG1 antibodies and other therapies (Figure 4), (24). Three doses of CD20-TCB were compared (0.5, 0.15, 0.05 mg/kg, IV all given once weekly). Whereas all doses mediated efficient B cell depletion and transient T cell decrease in the peripheral blood (Figure 4A), along with cytokine increase measured 24 h after the first TCB administration (Figure 4B; supplemental Table 4), only the intermediate
(0.15 mg/kg) and the highest (0.5 mg/kg) doses induced dose-dependent regression of established tumors (Figure 4C). Together, CD20-TCB treatment led to efficient B cell depletion in the peripheral blood, spleen, and lymph nodes along with tumor regression. At least 5–10-fold higher TCB doses were required for the regression of established tumor lesions compared with those required for normal B cell depletion in the peripheral blood.

**Obinutuzumab (Gazyva) pre-treatment mitigates cytokine-release syndrome associated with CD20-TCB administration**

We hypothesized that initial B cell depletion by obinutuzumab (Gazyva) pre-treatment (Gpt) prior to CD20-TCB administration could safely reduce T cell activation by TCB in peripheral blood or other normal tissues, thus reducing the risk of cytokine-release syndrome (CRS) and other potential side effects. This was evaluated by administering CD20-TCB (0.5 mg/kg, once weekly) after a single injection of obinutuzumab (10 mg/kg IV 7 days before CD20-TCB) in WSU-DLCL2-bearing HSC-NSG mice (Figure 5). The anti-tumor activity of CD20-TCB administered after Gpt was comparable to that of CD20-TCB alone, confirming the combinability of the two agents at these doses despite binding to the same CD20 epitope (Figure 5A). Histology analysis of tumors collected 7 days after CD20-TCB or Gpt administration showed that both treatments led to comparable increase of intra-tumor T cells (Figure 5B, left). In addition, CD20-TCB monotherapy and Gpt+CD20-TCB achieved the same intra-tumor T cell infiltration as demonstrated at study termination (Figure 5B, right).

Interestingly, whereas all treatments led to comparable B cell depletion in peripheral blood (Figure 5C, B cell panel), there was a clear difference in the transient decline
of T cell counts between the groups (Figure 5C, T cell panel). As reported earlier, T cells experienced a strong transient decline in peripheral blood following the first CD20-TCB administration, but their numbers did not change in obinutuzumab and Gpt+CD20-TCB treatment groups, suggesting that T cells are not affected by obinutuzumab treatment in mice. Most importantly, Gpt significantly reduced the cytokine peak associated with the first CD20-TCB administration (Figure 5D, supplemental Table 4). This finding was reproduced in toxicology studies in cynomolgus monkeys (Figure 5E), further confirming that Gpt enables a safe depletion of peripheral blood and secondary lymphoid organ B cells and prevents the strong T cell-mediated cytokine release associated with the first CD20-TCB administration.

**CD20-TCB combination with obinutuzumab beyond Gpt**

The combinability of CD20-TCB and obinutuzumab was further explored by combining the two antibodies for several administration cycles in two different DLBCL models, WSU-DLCL2 and OCI-Ly18 (Figure 5F). The synergy of this combination is evidenced by a rapid tumor regression in all animals and in both tumor models as compared to the corresponding single agents.

**Gpt versus SUD: superior safety and efficacy**

Gpt was compared to SUD, an approach frequently used in the clinic as a means of mitigating the CRS of the first TCB administration (Figure 6). SUD corresponded to 1/30 (0.015 mg/kg), 1/10 (0.05 mg/kg), and 1/3 (0.15 mg/kg) of the full CD20-TCB dose (0.5 mg/kg). Seven days after the SUD, all animals received the full CD20-TCB dose (0.5 mg/kg). Gpt was either superior (vs 0.015 mg/kg) or comparable (vs 0.05 and 0.15 mg/kg) to SUD doses in terms of the efficiency of peripheral blood and...
spleen B cell depletion (Figure 6A; supplemental Table 4). Importantly, Gpt was superior to SUD in terms of reducing cytokine release upon CD20-TCB administration, indicating that Gpt provides a safer B cell depletion approach than SUD (Figure 6B, left; supplemental Table 4). Moreover, administration of CD20-TCB full dose (0.5 mg/kg) 7 days after 0.015 and 0.05 mg/kg SUD led to a substantial cytokine peak, which was not observed with Gpt or the highest SUD tested (0.15 mg/kg), consistent with efficacious B cell depletion in peripheral blood during the first cycle by these two approaches (Figure 6B, right; supplemental Table 4). However, as the 0.15 mg/kg SUD was associated with significant cytokine release during the first cycle, Gpt provided an overall safer approach for CD20-TCB administration.

Gpt was also superior to all SUD groups in terms of anti-tumor activity. Whereas all treatment options led to comparable tumor growth inhibition (Figure 6C), there were more tumor-free animals (defined as those having tumor lesion size <10 mm$^3$ and not having malignant tumor cells assessed by histology) at study termination in the Gpt group (n=6) than in the SUD groups (n=4 in 0.015 mg/kg; n=2 in 0.05 mg/kg; n=4 in 0.15 mg/kg).

Histology analysis of lungs collected from mice 7 days post first treatment revealed a prominent perivascular T cell localization in all animals treated with SUD (0.15 and 0.05 mg/kg) but not with Gpt nor with the lowest SUD (0.015 mg/kg; Figure 6D, left; supplemental Table 5). Perivascular T cell localization persisted at later time points (24 h post-administration of the full CD20-TCB dose [0.5 mg/kg]) in all animals that received SUD, but not in the ones with Gpt (Figure 6D, right; supplemental Table 5). Conditioned media collected from tumor lysis experiments (containing a number of cytokines secreted by CD20-TCB-activated T cells, supplemental Figure 5A) induced
the activation of endothelial cells, as shown by strong upregulation of endothelial cell adhesion markers (E-selectin, VCAM, ICAM; Figure 6E&F). The same was true for mouse sera derived from a single treatment with CD20-TCB, but not from those receiving obinutuzumab (supplemental Figure 6A), consistent with the high levels of cytokines secreted in the peripheral blood upon CD20-TCB but not obinutuzumab administration (Figure 5D). In addition, CD20-TCB-activated T cells upregulated LFA-1 (ICAM-1 ligand; Figure 6G) and had a high constitutive expression of VLA-4 (VCAM ligand; supplemental Figure 6B), thus increasing T cell adhesion to CD20-TCB conditioned-media-activated endothelium (Figure 6H). Blocking experiments revealed that TNFα plays an important role in endothelial activation as TNFα-neutralizing antibodies significantly reduced upregulation of adhesion molecules by human endothelial cells (supplemental Figure 6C). Together, cytokines released by activated T cells during CD20-TCB-mediated B cell killing can induce endothelial activation and promote T cell adhesion. TNFα appears to play a key role in orchestrating this process.
Discussion

Despite generating promising clinical activity and offering curative solutions for a proportion of patients, T cell engaging approaches including TCB antibodies, are associated with severe side effects upon infusion requiring the introduction of SUD regimens to mitigate safety. Even newly-developed, half-life-extended TCB antibodies with IgG-like TCB 1:1 molecular format have similar issues, in addition to having a suboptimal format that hampers maximal potency.

Here we present a novel approach that addresses both short-comings. We developed a potent CD20-targeting TCB antibody (CD20-TCB) with a long half-life and strong potency enabled by head-to-tail orientation and high-avidity binding to CD20 in the 2:1 molecular format (21-23), along with a safer administration regimen for such potent drugs consisting of a single administration of obinutuzumab prior to the first infusion of CD20-TCB (Gazyva pre-treatment, Gpt).

CD20-TCB displayed significant potency and anti-tumor activity in vitro (even on cells expressing low levels of CD20), ex vivo (using tumor-infiltrated primary bone marrow aspirates of aggressive lymphoma and leukemia patients), and in vivo (where it regressed established tumors of an aggressive DLBCL model). Interestingly, potent killing activity was observed even at very low effector:target cell ratios, including those in primary tumor samples, which may be reflective of both the serial killing mediated by TCB-engaged T cells and of their rapid amplification as a result of TCB-mediated activation (25). The stronger potency of CD20-TCB relative to 1:1 CD20-TCB antibodies (some of which are in Ph I clinical development), highlighted the relevance of having the CD20 binder and CD3 binder in close proximity (enabled by a short flexible linker), as opposed to having them distant as in
the classical 1:1 IgG-based TCB format. Such superior killing potency has been observed for TCBs against other cancer cell targets and we hypothesize it may be related to a tighter and more stable T cell-cancer cell synapse induced by the head-to-tail fusion molecular format (26).

The increased potency of CD20-TCB over other TCBs increases the risk of side effects due to higher levels of cytokine release resulting from stronger T cell activation and more potent B cell killing. If not adequately mitigated, this may lead to severe toxicity and limit the doses required for efficacious tumor control. Steroid pretreatment is used clinically to reduce systemic T cell activation and cytokine release; however, it is currently unclear whether high-dose and prolonged steroid treatment might affect T cell activation and functionality (12, 27-29). Alternative clinical approaches include use of the IL-6 receptor blocking antibody (tocilizumab) or fractionation of the first TCB dose (so-called SUD approach). Here we present an alternative and novel approach consisting of a single obinutuzumab pretreatment (Gpt) 7 days prior to the first CD20-TCB administration. This approach was both more efficacious than SUD, achieving greater B cell depletion (de-bulking) in both peripheral blood and secondary lymphoid organs, but also increased safety, as it abrogated the initial strong cytokine release associated with T cell activation. Such T cell activation during peripheral blood and normal tissue B cell depletion, makes SUD inherently less robust as a pretreatment approach, as evidenced by the proportion of patients that still experience significant side effects after SUD (12). Because of lower cytokine release, obinutuzumab pretreatment also prevented the transient T cell margination and extravasation into peripheral tissues, possibly via reducing cytokine-mediated endothelial cell activation.
Importantly, despite competing for the same target antigen, obinutuzumab and CD20-TCB could be combined in vitro and in vivo, yielding promising anti-tumor efficacy. The 2:1 TCB molecular format of CD20-TCB provides two CD20 binding Fabs (as in obinutuzumab), thus conferring to the two molecules the same binding avidity to CD20 antigen, enabling CD20-TCB binding even in presence of saturating concentrations of obinutuzumab. Indeed, the same avidity for CD20 confers the possibility for CD20-TCB to outcompete pre-bound obinutuzumab; which is a significant challenge for 1:1 IgG CD20-targeting TCB antibodies that have only a single low affinity CD20 binding Fab. This may be relevant in future front-line settings where CD20-TCBs might be combined with standard of care therapies with a rituximab or obinutuzumab backbone (e.g., R-CHOP in DLBCL; obinutuzumab in CLL; obinutuzumab+chemotherapy in FL). Combining T cell-redireced and IgG1-antibody-mediated approaches is attractive in cancer treatment due to synergy and complementarity of different modes of action that combine T cell-mediated cytotoxicity (of TCB antibodies) with ADCC, phagocytosis (30, 31), complement-dependent-cellular cytotoxicity, and direct cell death induction of type II anti-CD20 antibodies. Consistent with this, the sustained and simultaneous combination of CD20-TCB and obinutuzumab for several treatment cycles did not affect CD20-TCB activity and translated into a rapid and more profound anti-tumor efficacy with tumor regression in all animals as compared to single agent treatment arms.

In conclusion, we present a novel, more efficacious and safer approach for treatment of NHL, consisting of a single fixed dose of obinutuzumab prior to CD20-TCB therapy. This approach is currently under evaluation in a Phase I, multicenter, open-label, dose-escalation study in patients with relapsed/refractory NHL (NCT03075696). Clinical data will clarify whether the strong efficacy seen in pre-
clinical models translates into clinical response and more favorable safety compared to approaches currently used in the clinic.
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References


7. Younes A, Thieblemont C, Morschhauser F, Flinn I, Friedberg JW, Amorim S, et al. Combination of ibrutinib with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) for treatment-naive patients with CD20-


Figure legends

**Figure 1.** *In vitro* and *ex vivo* tumor cell lysis mediated by different CD20-TCB variants. (A) Tumor cell lysis mediated by 2:1 CD20-TCB, 1:1 IgG CD20-TCB and 1:1 OA CD20-TCB, determined using LDH release after 20–24 h incubation of human PBMCs with tumor targets and indicated TCB concentrations (E:T 5:1). One representative donor of total 3 donors is shown (other donors are presented in supplemental Figure 2B). (B) Tumor cell lysis mediated by obinutuzumab-based 2:1 CD20-TCB as compared to 1:1 CD20-TCB having alternative anti-CD20, anti-CD3, and Fc sequences (VH-A-1242-1250; WO2014/047231 A1). One representative donor of total 3 donors is shown (other donors are presented in supplemental Figure 2B). (C) EC$_{50}$ values of 2:1 CD20-TCB-mediated tumor cell lysis in bone marrow aspirates derived from patients with aggressive lymphoma and leukemia (n=17 patients). Each dot represents the average EC$_{50}$ per patient derived from different time points (24–120 h). (D) Examples of concentration-dependent B cell depletion curves mediated by 2:1 CD20-TCB in three different patients (P4, P8, P17).
Figure 2. Mode of action of CD20-TCB. (A) In vitro visualization of the dynamics of CD20-TCB (white) during interactions between CD8+ T cells (green) and WSU DLBCL tumor cells (blue). Image sequence highlights CD20-TCB localization in immunological synapse areas along with tumor cell killing evidenced by the formation of tumor cell blebs. CD20-TCB was fluorescently-labeled using Alexa Fluor 647 (white signal). (B) Quantification of T cell speed, synapse area and CD20-TCB intensity at synapse. (C) Flow cytometry analysis of samples collected within 1–3 days of tumor cell lysis show the kinetics of expression of T cell activation (CD25, 4-1BB, Ox40, ICOS) and exhaustion markers (PD-1, Lag3).
Figure 3. In vivo activity of CD20-TCB. (A) Non-tumor bearing fully humanized NSG mice injected with CD20-TCB (0.5 mg/kg) or with phosphate saline buffer (PBS, Vehicle) at study day 0 and day 7. Mice were bled at baseline (i.e., prior to the first CD20-TCB injection) and at indicated time points for pharmacokinetic (PK) and pharmacodynamics (PD) analysis. (B) Kinetics of peripheral blood B cells and T cells counts. Flow cytometry analysis of CD19\(^+\) B cells (left panel) and CD3\(^+\) T cells (right panel) in blood of vehicle- and CD20-TCB-treated mice (0.5mg/kg) over time. (C) PK results of CD20-TCB in fully humanized and non-humanized NSG mice after CD20-TCB single dose injection (0.5 mg/kg). Single mice are shown for each of the two mouse models (n=3). (D) T cell phenotype analysis in the peripheral blood at 72 h post first CD20-TCB injection. Flow cytometry analysis of CD25, 4-1BB, PD-1, Ki-67, and GZB on CD4\(^+\) and CD8\(^+\) T cells. Frequency of marker-positive cells are reported as % for vehicle- (grey) and CD20-TCB-treated mice (red). (E) Multiplex analysis of cytokines in blood of vehicle and CD20-TCB-treated mice over time displaying the intensity of cytokine release at 24 h and 72 h post first and 72 h post second CD20-TCB injection (labeled as 1d, 3d, 10d respectively). The absolute values of cytokines are reported in supplemental Table 4. (F) Immunohistochemical analysis (CD19 staining) of human B cells in spleen and lymph nodes (LN) of vehicle and CD20-TCB-treated mice at study termination (d10 corresponding to 3 days after the second CD20-TCB injection).
Figure 4. In vivo anti-tumor activity in a DLBCL xenograft model. (A) Flow cytometry analysis of CD19+ B cells and CD3+ T cells in peripheral blood of vehicle and CD20-TCB-treated mice (0.5 mg/kg, 0.15 mg/kg, 0.05 mg/kg IV once weekly) measured 24 h post first administration. Baseline indicates the level of B cell and T cell counts in mice prior to treatment. (B) Multiplex analysis of cytokines released in the peripheral blood of vehicle- and CD20-TCB-treated mice detected 24 h after the first administration. The absolute values of cytokines detected are reported in supplemental Table 4. (C) Anti-tumor activity of CD20-TCB at different doses (0.5 mg/kg, 0.15 mg/kg, 0.05 mg/kg IV once weekly) in fully humanized NSG mice bearing WSU-DLCL2 (DLBCL) xenografts. Red arrows indicate therapy injection once per week (n=10 animals/group).
Figure 5. Effect of obinutuzumab (Gazyva) pre-treatment (Gpt) on T cell activation by CD20-TCB and cytokine release. (A) Anti-tumor activity of vehicle; CD20-TCB monotherapy (0.5 mg/kg IV once weekly); obinutuzumab (Gazyva) monotherapy (10 mg/kg IV once weekly); and obinutuzumab (Gazyva) pretreatment (Gpt, 10 mg/kg single injection) followed by CD20-TCB (0.5 mg/kg IV once weekly) in WSU-DLCL2-tumor bearing fully humanized NSG mice. Arrows indicate therapy injections (red arrow: CD20-TCB; blue arrow: obinutuzumab; number of animals/group 8 < n < 10). (B) Immunohistochemical staining showing intra-tumor T cell infiltration (CD3 staining, brown) at indicated time points and for indicated groups. Histograms denote the quantification of the intra-tumor CD3 T cell infiltrate among the various groups. (C) Flow cytometry analysis of CD19+ B cells (left) and CD3+ T cells (right) in the peripheral blood of vehicle and CD20-TCB-treated WSU-DLCL2-tumor bearing fully humanized NSG mice, 24 h and 72 h after first and second therapy injection as indicated by the legend. (D) Cytokines released in peripheral blood among the different treatment groups 24 h after the first and second treatments. The absolute values of cytokines detected are reported in supplemental Table 4. (E) Toxicology study in cynomolgus monkey with administration of vehicle or obinutuzumab (50 mg/kg IV) at day 1 to reduce B cell load. Five days later animals were either treated with vehicle or CD20-TCB (0.1 mg/kg IV) and IFNg in plasma was measured after 4 h. (F) Anti-tumor activity obtained upon CD20-TCB, obinutuzumab (Gazyva) or CD20-TCB+obinutuzumab (Gazyva) treatment in WSU-DLCL2 and OCI-Ly18 DLBCL tumor models in HSC-NSG mice. Doses are indicated on figure panels; the number of injections was n=7 for WSU-DLCL2 and n=2 for OCI-Ly18. All therapies were administered once weekly; the combination of CD20-TCB and obinutuzumab was performed by concomitant administration of both antibodies.
at each treatment cycle. Red arrow indicates the start of therapy injection; treatments started at tumor size of 520-550 mm³ in the WSU-DLCL2 tumor model (study day 15 upon tumor-cell inoculation), and 360-380 mm³ in the OCI-Ly18 tumor model (study day 15 upon tumor-cell inoculation). Number of animals/group, 8 < n < 10.
Figure 6. Safety and efficacy of CD20-TCB with obinutuzumab pretreatment (Gpt) or as SUD in tumor-bearing WSU-DLCL2 HSC-NSG mice. Comparison of obinutuzumab pretreatment (Gpt) to step-up dosing (SUD) approach in tumor-bearing WSU-DLCL2 (SC) in HSC-NSG mice. Mice received the first therapy either as SUD of CD20-TCB (0.15, 0.05, 0.015 mg/kg IV) or obinutuzumab pretreatment (Gpt, 10 mg/kg), followed by a full dose of CD20-TCB (0.5 mg/kg) 7 days later for 5 treatment cycles (i.e., 5 weeks). (A) Flow cytometry analysis of CD19+ B cells and CD3+ T cells in the peripheral blood and spleen of vehicle, SUD and Gpt-treated WSU-DLCL2-tumor bearing HSC-NSG mice, 7 days post first therapy injection. (B) Cytokines released in peripheral blood among the different treatment groups 24 h after the first and second treatments. The absolute values of cytokines detected are reported in supplemental Table 4. (C) Anti-tumor activity of SUD versus Gpt + CD20-TCB approach. Arrows indicate therapy injections (red arrow: CD20-TCB; blue arrow: obinutuzumab). Number of animals/group (n=9 or 10). (D) Perivascular T cell localization in lungs of WSU-DLCL2 tumor-bearing HSC-NSG mice 7 days after the first treatment and 24 h after the second treatment. Lung sections were immunohistochemically-stained with anti-CD3 antibody (brown); nuclei were counterstained with hematoxylin. Magnification 20x. Supplemental Table 5 shows the average score of perivascular CD3 positive T cells in the lung (performed by blinded pathologist analysis). (E) Schematic representation of the assay used to test the effect of CD20-TCB conditioned media from tumor lysis experiments on endothelial cell activation and T cell adhesion. (F) Human endothelial cells (HUVEC) were stimulated for 6 h with conditioned media from tumor lysis experiments (PBMCs plus Z-138 tumor cells incubated with CD20-TCB or untargeted TCB for 24 h) and adhesion molecules were measured by flow cytometry. CD20-TCB
conditioned media potently upregulated ICAM, VCAM and E-selectin. TNFα (10 ng/mL) was used as positive control. Data is representative of >3 experiments. (G) Activated T cells upregulate LFA-1 expression upon CD20-TCB-mediated tumor lysis. (H) T cells isolated from tumor lysis experiments in presence of CD20-TCB were fluorescently labeled and let to adhere for 1 h on HUVEC monolayers stimulated with conditioned media collected from the same tumor lysis experiment as depicted in panel (E). T cell adhesion (measured as fluorescence units, F.U.) was increased on endothelium stimulated with CD20-TCB conditioned media as compared to untargeted TCB conditioned media. Data is representative of two independent experiments. Statistical analysis: One-way ANOVA.
Figure 1

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CD20-TCB with obinutuzumab pretreatment as next generation treatment of hematological malignancies

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