APPLICATION NOTE

Optimization of complement-dependent cytotoxicity assay on OncoSignature cell lines.

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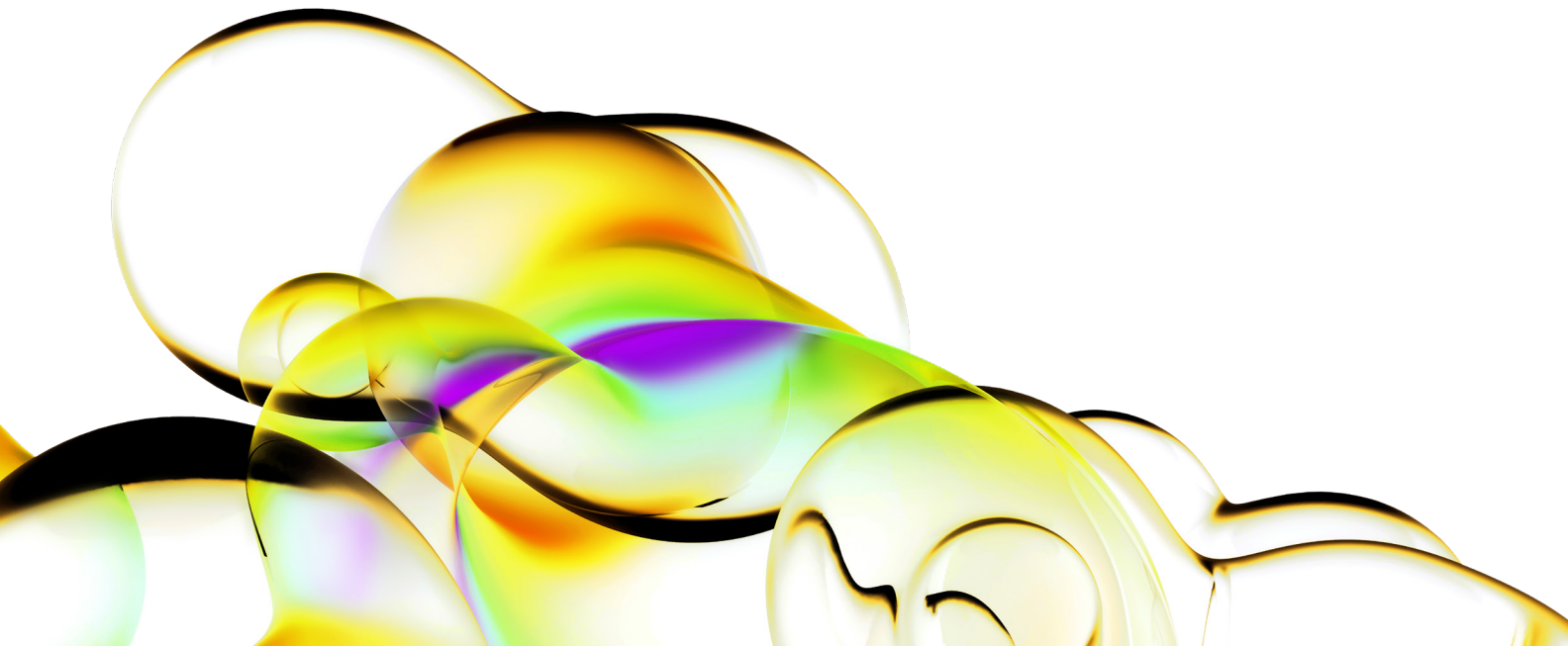
Introduction

Therapeutic monoclonal antibodies (mAb) are powerful tools for treating cancers and autoimmune diseases. Through their ability to specifically bind cell surface proteins, mAbs can modulate signaling in tumor and immune cells, or trigger responses through the activation of complement and engagement of antibody receptors on immune cells [1]. Complement-dependent cytotoxicity (CDC) is an example of an immune response in which target cells are lysed through activation and recruitment of complement to the cell surface. CDC is a tightly regulated process, and several therapeutic mAbs utilize the CDC mechanism with varying degrees of success [2]. Ultimately, many factors play a role in determining CDC efficiency: antibody isotype, antigen location, binding geometry, Fc receptor affinity, the expression level of the target antigen, and expression of complement regulatory proteins [3] [4] [5]. Therefore, screening candidate mAbs for CDC activity represents a crucial step in the development pipeline of successful therapeutics. Here we present how our CDC assay offering can help drug screening efforts by leveraging our semi-automated screening platform and OncoSignature™ cell panel.



Assay overview

We performed a CDC assay screen using the therapeutic mAb rituximab. Rituximab, first approved by the FDA in 1997, is one of the clinic's first and most successful therapeutic mAb and is regularly used in treating a range of B-cell malignancies [6]. The antibody is also well characterized and is thus used to evaluate the performance of our in-house CDC assay. The CDC assay uses calcein release as a readout of cell lysis. Calcein-AM is a cell-permeant, non-fluorescent dye that, when taken up by live cells, is converted by intracellular esterases into a green fluorescent dye. Cells undergoing lysis release the dye into the supernatant, providing a measure of cell death. The assay also incorporates semiautomation for all cell seeding and liquid handling steps to provide high accuracy and assay reproducibility. Semi-automation also enables the assay to be scaled up to meet the demands of more extensive research programs. In the following, we describe the workflow and the results of the CDC assay obtained (Figure 1).

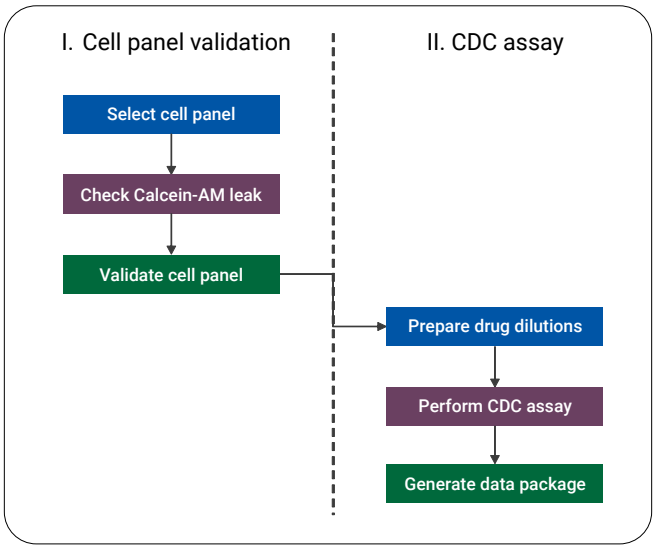


Figure 1: Schematic of the workflow of the CDC assay. It consists of two phases, the validation of the cell lines' ability to release calcein, and a second stage where the testing compounds challenge the selected cell lines.

Selection of a cell line panel

As the first step in our workflow, we selected and validated a selection of cancer cell lines from the OncoSignature™ library of standard cancer cell lines. Since the efficiency of cell lysis depends on target expression on the cell surface, and considering that rituximab binds to the CD20 receptor and promotes lysis of CD20-expressing cells, we selected cell lines with varying levels of CD20 based on protein expression data obtained from the publicly available CCLE database [7]. We curated our in-house OncoSignature™ cell line panel and selected seven cell lines with different levels of CD20 expression (Figure 2a). We then assessed CD20 expression in the seven cell lines using flow cytometry and confirmed that the relative surface expression levels were comparable to gene expression levels obtained from the CCLE database (Figure 2b). Based on the excellent correlation in target expression between the published data in the CCLE database and our in-house flow cytometry results, interrogation of the proteomics CCLE database prior to CDC assay setup has become an integral part of our workflow to identify suitable cell lines candidates for the assay.

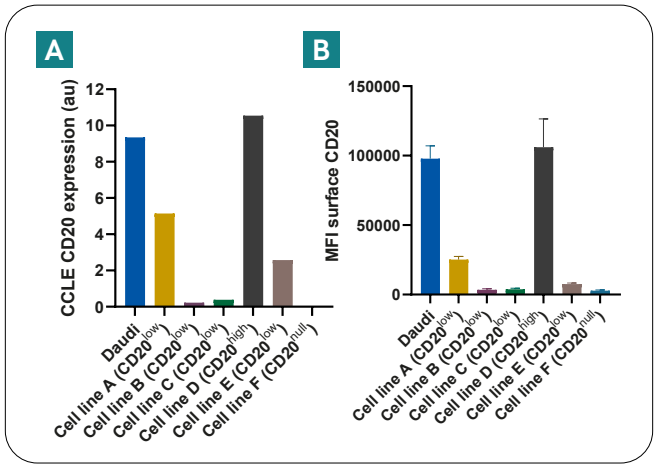


Figure 2: Expression of surface CD20 in selected cell lines. A: CD20 expression data for the seven listed cell lines obtained from the proteomics data set from the Cancer Cell Line Encyclopedia (CCLE). B: Mean fluorescence intensity (MFI) values of cells stained with α-CD20-APC and analyzed by flow cytometry showing the expression levels of CD20 in the seven listed cell lines.

Evaluation of calcein retention

Calcein does not bind to any intracellular components, and spontaneous leakage occurs depending on the inherent properties of a particular cell type or cell culture conditions. Therefore, assessing the baseline calcein retention in our seven cancer cell lines panel is critical before proceeding with the CDC assay. Briefly, cells were loaded with calcein-AM and incubated at 37 °C for the same period as the CDC assay before measuring the level of fluorescence in the supernatant. To measure maximal calcein release, we treated calcein-AM-loaded cells with 0.1% saponin

to simulate total cell lysis. The seven cell lines tested showed different levels of maximal calcein release in the saponin-treated cells at identical cell numbers seeded, indicating that the level of calcein-AM loading is cell line dependent.

Similarly, the level of spontaneous calcein released in the supernatant by calcein-AM stained cells differed between the cell lines (Figure 3a). We plotted the relative spontaneous calcein released for each cell line by expressing the supernatant fluorescence of calcein-AM stained cells as a percentage of the supernatant fluorescence of saponintreated cells (Figure 3b). Most cell lines tested showed low background levels (20-30%) of spontaneous calcein release, with only two cell lines releasing close to 50% of loaded calcein-AM (dotted line in Figure 3b). We set 50% as the threshold for identifying cell lines unsuitable for the CDC assay. Based on the results from our validation experiments, we chose five cell lines as our test panel for the CDC assay setup, including two CD20^{high} cell lines: Daudi and Raji cells, two CD20^{low} cell lines and one calcein "leaky" cell line.

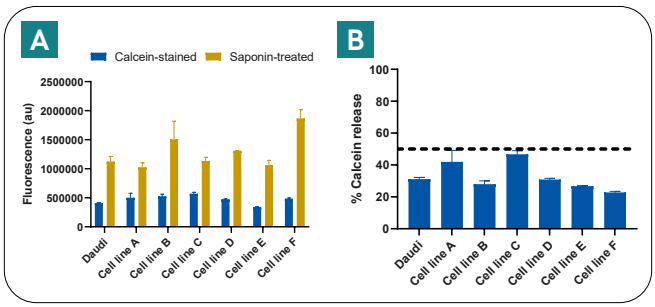


Figure 3: Calcein release threshold determined in cell lines. Fluorescence of. supernatants from cells stained with calcein-AM and treated with 0.2% saponin (gold bars) or left untreated (blue bars) for the seven cell lines listed. B: Percentage calcein release for the seven tested cell lines described in the defined materials and methods section. The dashed line marks the 50% threshold over which cells are considered "leaky" for calcein staining.

CDC assay performance

We next took the panel of five cell lines and performed a CDC assay to test the activity of two therapeutic mAbs: rituximab and a rituximab biosimilar (referred to as biosimilar). Using rituximab, which is widely considered the gold standard for treating human B cell malignancies, we aimed to showcase the ability of our CDC assay workflow to quantify complement-dependent cell killing as well as highlight our capability to compare different therapeutic mAbs across multiple cell lines directly.

In brief, cells were loaded with calcein-AM and incubated with either rituximab or biosimilar at different concentrations before adding human serum. Following incubation in the presence of serum and collection of the supernatant, we assessed the cell lysis by measuring calcein release by calculating the fluorescence of the supernatant of mAbtreated cells as a percentage of the fluorescence of the supernatant of the saponin-treated cells. The obtained values were then plotted over the concentration of therapeutic mAb to generate dose-response curves (Figure 4). As negative controls, we included cells treated with a non-specific IgG (isotype control) and used incubation with heatinactivated (HI) serum.

We observed a similar pattern when comparing the activity of rituximab or its biosimilar across the five cell lines. Raji and Daudi cells showed the highest level of cell killing for both antibodies, with higher levels of cell lysis detected in the former compared to the latter, while the other three cell lines showed little to no cell lysis. These results were anticipated as both Raji and Daudi cells express CD20 at very high levels. The data from our comparison between these two mAbs further underlines how critical antigen expression levels are for effective CDC lysis. It also highlights how antibodies induce varying levels of CDC lysis efficiencies relative to the level of target antigen expression on cells.

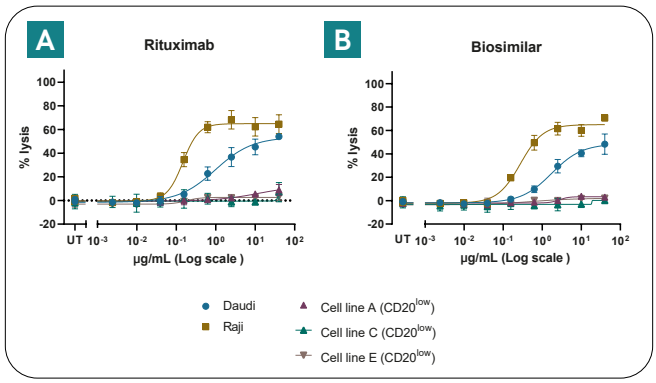


Figure 4: Rituximab and its biosimilar show similar complement-dependent lysis percentages. CDC assay performed in the five cell lines listed and treated with rituximab (A) or rituximab biosimilar (B). Percent lysis plotted over drug concentration. UT: Untreated.

Using the same CDC assay dataset, we also compared how the different cell lines responded to rituximab or the biosimilar. Here, we highlight Daudi and Raji cells as the most susceptible cell lines to CDC. In both cell lines, rituximab performed marginally better than the biosimilar, as demonstrated by its lower EC50 values (0.147 µg/mL rituximab vs 0.275 µg/mL biosimilar in Raji cells, 0.985 µg/mL rituximab vs 1.746 µg/mL biosimilar in Daudi cells).

The difference in EC50 values was more significant at lower mAb concentrations while the two mAbs performed identically at higher concentrations. Cells treated with IgG control or HI serum showed no lysis. (Figure 5a-b). Cell line A cells showed negligible levels of cell lysis, despite expressing CD20 at a substantially higher level than the Cell line C and Cell line E, which revealed no cell killing (data not shown). However, we could detect a low percentage of cell lysis, above that of the assay controls, in cells treated with the highest dose of rituximab (Figure 5c). This observation highlights the sensitivity of our assay and our ability to measure a percentage cell lysis level as low as 10%.

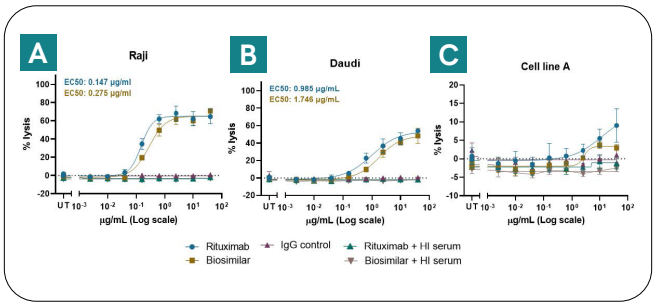


Figure 5: A sensitive complement-dependent lysis assay compares compounds over high-antigen-expressing cell lines. Percentage lysis in Raji cells (A), Daudi cells (B), and Cell line A (C) treated with rituximab, biosimilar, IgG control, rituximab and HI serum, biosimilar and HI serum plotted over drug concentration. A and B: EC50 values for rituximab (blue) and biosimilar (gold) were calculated in GraphPad Prism using a sigmoidal four-parameter logistic curve. UT: Untreated.

Our results using the calcein "leaky" Cell line C highlighted important caveats for studying such low levels of CDC activity (Figure 6). We observed that Cell line C displayed a high degree of variability in the cell lysis percentage, independent of antibody concentration used. Such high variability could mask subtle changes in CDC activity for a particular cell line. To better understand this observation in the cell line C, we plotted the percentage of cell lysis in the presence of IgG control antibody and HI serum against IgG antibody concentration (Figure 6). The data revealed that the high variability of percentage lysis in cell line C persisted even in the presence of IgG control and HI serum, while all other cell lines showed no CDC lysis (Figure 6e). As no cell killing agent was present in the experimental conditions for these cells, we concluded that leaked calcein is the likely source of variability. This observation emphasizes the importance of validating cell lines by testing the level of calcein leakage prior to performing a CDC assay.

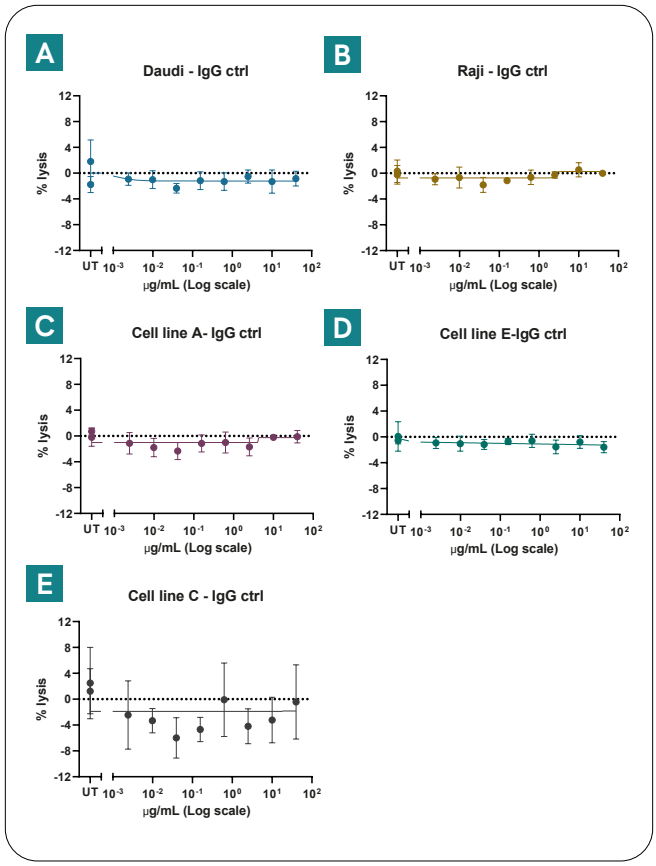


Figure 6: Cell lysis with unspecific antibodies demonstrates the importance of calcein leakage assessment. Percentage cell lysis in Daudi cells (A), Raji cells (B), Cell line A (C), cell line E cells (D), and cell line C (E) treated with IgG control and HI serum plotted over control IgG concentration. UT: Untreated.

CDC assay outlook

Our standard CDC assay offering depicted in this application note has shown examples of the data end users can obtain. In addition, it is essential to highlight that the standard CDC platform can offer additional information for the therapeutic mAb development field. When selecting a cell line panel for the CDC assay, we propose to include OncoSignature™ cell lines with a broad range of target expression levels, similarly to our CD20 assay presented above, i.e., low, mid, and high expression. This approach enables the creation of an activity profile for each therapeutic mAb tested, highlighting how given concentrations of mAb respond to specific expression levels of the target antigen (Figure 7). Such data could prove key in refining therapeutic mAb dosing or comparing the effectiveness of distinct mAbs at different antigen expression levels.

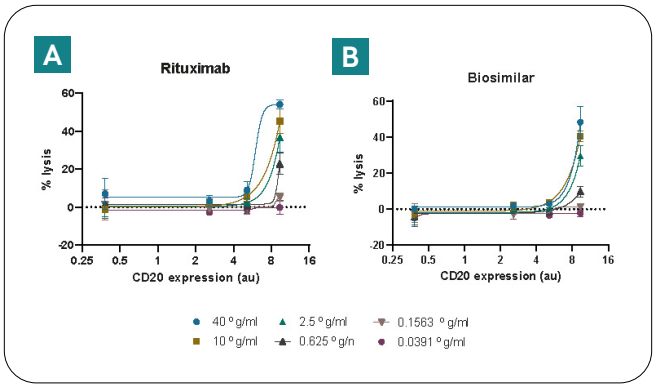


Figure 7: CDC assay allows relating the activity profile of the therapeutic mAb to the specific expression levels of the target antigen. Activity profile of rituximab (A) and biosimilar (B). Percentage cell lysis of the drugs at the listed concentrations was plotted over the expression level of CD20 (au = arbitrary units), as obtained from the proteomics dataset from CCLE for the five cell lines used in this study.

CDC killing efficiency depends on the therapeutic mAbs and the expression of complement regulatory proteins. These proteins are often expressed or upregulated by target cancer cells and generally inhibit CDC activity in the tumor environment [8]. Several studies have shown that the blocking of these complement regulators can enhance the CDC activity of therapeutic antibodies, thus proving to be an attractive opportunity for the development of combination therapies that can enhance CDC activity [9, 10, 11, 12]. Our CDC assay offers a platform to screen such drugs for their ability to modulate CDC killing by therapeutic mAbs. An initial CDC assay using a chosen therapeutic mAb can allow finding a sub-saturating mAb concentration to detect increases in CDC activity. Then a second CDC assay is performed, in which cells are treated with the previously determined concentration of therapeutic mAb and titration of the candidate CDC-modulating drug, thus enabling the identification of potential CDC-enhancing compounds or possible drug-therapeutic mAb combinations.

Concluding remarks

The CDC assay is a powerful screening platform to aid the development of successful therapeutic mAbs. Here, we have developed a robust CDC assay workflow incorporating semi-automation, which accurately and reproducibly supports the testing and comparison of mAbs candidates across a panel of cancer cell lines. Leveraging OncoSignature™ cell line panel, we can apply our CDC assay workflow to test mAbs targeting a broad range of surface proteins with various expression levels, thus ensuring that candidate mAbs can be validated across several cell lines and lineages.

Methods

Flow cytometry staining

Cells were washed twice with PBS and stained with α-CD20-APC (Cat #302310, BioLegend) diluted 1:400 in PBS. After 15 min incubation at room temperature, samples were acquired using an iQue PLUS flow cytometer and data were analyzed using IntelliCyt ForeCyt 9.0. Mean fluorescence intensities were plotted using GraphPad Prism 9.3.0. CD20 expression data was obtained from the Proteomics data set from the Cancer Cell Line Encyclopedia (CCLE, Broad Institute) and plotted using GraphPad Prism 9.3.0.

Calcein retention

Resuspended cells at $1x10^6$ cells/mL density in basal media were treated with 5 µM calcein-AM for 30 min at 37 °C. Then, basal media was added for a further 10 min at 37 °C incubation. After centrifugation, the pellet was washed twice with PBS before resuspending in complete media and plating into the assay plate in quadruplicates. For the saponin-treated control samples, cells were loaded with calcein-AM as above, plated, and 0.2% (v/v) saponin was added in a complete media solution. For unstained control samples, we used calcein-AM untreated cells incubated for 2 h at 37 °C, and pellets and supernatants were transferred to a reading plate. The EnVision8 plate-reader (Revvity, Inc) measured fluorescence, and values were plotted as bar graphs using GraphPad Prism 9.3.0. The percent calcein release was calculated as the percent supernatant fluorescence of calcein-AM stained samples over the supernatant fluorescence of calcein-AM stained cells treated with 0.2% saponin.

CDC assay

An eight-point, four-fold dilution series of rituximab and a rituximab biosimilar were prepared to start from a maximum concentration of 40 µg/mL. Cells were stained with calcein-AM as described above and seeded into an assay plate containing the compound dilutions, then incubated for

30 min at 37 °C. At an optimized concentration, whole or heat-inactivated human serum was added to defined wells, and the cells were incubated for another 2 h at 37 °C. After centrifugation, supernatants were transferred to a reading plate. The level of fluorescence in the supernatants was analyzed using an EnVision8 plate-reader (Revvity, Inc).
The fluorescence of supernatants from cells incubated with calcein-AM and serum, but without a mAb, was subtracted from all samples and controls to correct for background fluorescence resulting from the assay media or spontaneous calcein release. Background-corrected values were used to calculate the percentage of cell lysis, which was defined as the per cent supernatant fluorescence of the test sample over the supernatant fluorescence of saponin-treated.

$$\text{Percent lysis} = \frac{\text{Fluorescence sample} - \text{Fluorescence Untreated}}{\text{Fluorescence saponin sample} - \text{Fluorescence Untreated}} * 100$$

Activity profile plots were generated by plotting the percentage cell lysis over CD20 expression levels as obtained from the Cancer Cell Line Encyclopedia (CCLE, Broad Institute) using GraphPad Prism 9.3.0 for data visualization.

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