Generation and Validation of Anti-CD19 Single Chain Variable Fragment-Hydrophobin SpyTag-SpyCatcher Protein System for Targeted Drug Delivery to B-Cell Acute Lymphoblastic Leukemia

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Lauren Harkins
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Dr. Bryan Berger, Department of Chemical Engineering

Generation and Validation of Anti-CD19 Single Chain Variable Fragment-Hydrophobin SpyTag-SpyCatcher Protein System for Targeted Drug Delivery to B-Cell Acute Lymphoblastic Leukemia

Lauren Harkins[a], Nathanael Sallada[a], Bryan Berger[a,b,1]

[a]Department of Biomedical Engineering, University of Virginia
[b]Department of Chemical Engineering, University of Virginia
[1]Correspondence:
Email: bryan.berger@virginia.edu
Address: Thornton Hall, P.O. Box 400259, Charlottesville, Virginia 22904
Phone: 434-924-2016

Abstract:

Currently, there is a lack of inexpensive and effective targeted treatments for B cell acute lymphoblastic leukemia (B-ALL), a condition where the body accumulates malignant lymphoid cells. We are proposing a novel drug delivery mechanism that can target the lymphoid cells. This drug delivery mechanism is a protein system composed of hydrophobin (HFBI) and an anti-CD19 short chain variable fragment (FMC63scFv) linked by the SpyCatcher-SpyTag mechanism. HFBI serves to solubilize the hydrophobic anti-cancer drugs, while the FMC63scFv targets the anti-cancer drugs to the lymphoid cells. The ability of HFBI to solubilize anti-cancer drugs while linked to the FMC63scFv would have been evaluated with emulsion stability test. The ability of FMC63scFv to target CD19 positive lymphoid cells will linked to the HFBI would have been evaluated using flow cytometry. The ability of the protein system to kill target cells while not being toxic to non-target cells would have been evaluated using lactate dehydrogenase (LDH) cell viability assays. Both FMC63scFv-SpyCatcher003 and SpyTag003-HFBI proteins were successfully produced recombinantly in *Pichia pastoris*. Preliminary results suggest that the FMC63scFv and HFBI proteins can be linked via the SpyTag-SpyCatcher mechanism, but further experiments need to be performed to confirm. Due to the inability to return to lab, we were unable to complete the functionality experiments to make a conclusion about whether the FMC63scFv-HFBI protein system can serve as a potential drug delivery system for B-ALL treatment.

Keywords: HFBI, FMC63scFv, SpyTag-SpyCatcher

Introduction:

B Cell Acute Lymphoblastic Leukemia (B-ALL)

B-ALL is characterized by the accumulation of lymphoid progenitor cells in the bone marrow and other tissues[1]. There are approximately 5000 new cases of B-ALL and over 1000 deaths due to B-ALL in the United States each year[2]. The long-term remission rate for adult patients with B-ALL is only between 30 and 40 percent[2]. This low rate of remission is due to the lack of inexpensive and effective targeted treatments. A common current treatment for B-ALL is chemotherapy[3]. While the chemotherapy is effective in killing cancerous cells, it is a systemic treatment that can harm healthy cells in the body as well[3]. Additionally, many of the anti-cancer drugs used are hydrophobic, so they require a chemical surfactant to solubilize them for delivery[4]. These chemical surfactants can cause immunogenic reactions in the already immunocompromised patients[4]. Another possible treatment method for B-ALL is chimeric antigen receptor (CAR)-T cell therapy against CD19, as the lymphoid cells that are accumulating are CD19 positive[2]. Unlike chemotherapy, CAR-T cell therapy is targeted, as it will only bind to cells that express CD19[5]. However, CAR-T cell therapy is extremely expensive. A single treatment costs $475,000, not including other expenses the patient might incur from hospital stays and any complications[5]. To improve the efficacy of the CAR-T cell therapy, allogeneic stem-cell transplantation may be done in conjunction with the first dose which costs an additional $200,000[5]. Furthermore, about half of patients will experience a relapse and need more treatment[5]. Other issues with CAR-T cell therapy include limited locations

performing this treatment, the 22 day time period to make the cells, and the risk of toxicity[5].

Hydrophobin

Hydrophobins are amphiphilic proteins characterized by four conserved disulfide bonds and high surface activity[4]. This high surface activity allows hydrophobins to create stable emulsions containing hydrophobic drugs[4]. Previous research has shown these proteins to be non-immunogenic, therefore being preferable over traditional chemical surfactants for drug delivery[6]. HFBI, a class II hydrophobin, has previously been shown to solubilize hydrophobic drugs by self-assembling around the hydrophobic drugs due its amphiphilic nature[4]. HFBI's function as a biosurfactant has been shown to depend on its four disulfide bonds[7]. When the HFBI is internalized into the cell, the reducing environment of the cytoplasm will cause the disulfide bonds to break[4]. With the breaking of these bonds, the proteins will no longer self-assemble around the hydrophobic drug, and the drug will then be released within the cell[4]. Therefore, the surrounding cells should not be affected by the anti-cancer drug as the drug is only released inside the cell[4].

Anti-CD19 Single Chain Variable Fragment

The anti-CD19 single chain variable fragment (scFv) used in this study was FMC63scFv. FMC63scFv was chosen as it targets human CD19, which is expressed on the lymphoid cells in B-ALL[8]. CD19 was chosen as a target because CD19 is only expressed on B cells, and the possible destruction of B cells is well tolerated[9]. Additionally, CD19 is not expressed on pluripotent blood stem cells or most other normal tissues in the body[9]. Using an scFv over the full anti-CD19 antibody was chosen because scFvs have reduced immunogenicity, lower retention time in non-target tissues, lower manufacturing cost, and quicker penetration in tissue compared to full antibodies[8,10].

SpyTag-SpyCatcher System

The SpyTag-SpyCatcher system was derived from a mechanism to stabilize extracellular proteins in gram positive bacteria[11]. In this system, the two proteins will spontaneously form an amide bond between the two proteins that is specific and irreversible[11]. This bond will form between the lysine and the aspartic acid as the unprotonated amine nucleophilically attacks the carbonyl carbon[11]. Keeble et al optimized the SpyTag-SpyCatcher system to create the amide bond faster and labeled the system SpyTag002-SpyCatcher002[12]. However, this sequence included a Kex2 site in both SpyCatcher002 and SpyTag002 that would be cleaved when the protein was made recombinantly in yeast. To avoid unwanted cleavage, the Kex2 site of lysine-arginine was changed to lysine-proline in SpyTag002 to make SpyTag003 and lysine-lysine in SpyCatcher002 to make SpyCatcher003. SpyTag003 is linked to HFBI. HFBI is a low-yield protein, so it is attached to the smaller of the two proteins to hopefully prevent any further decrease in protein yield[13]. FMC63scFv is linked to SpyCatcher003 with a flexible linker amino acid sequence. The complete protein amino acid sequences for both FMC63scFv-SpyCatcher003 and SpyTag003-HFBI can be seen in Figure S1.

Materials and Methods:

Plasmid synthesis

FMCscFv63-SpyCatcher003 and SpyTag003-HFBI inserts were constructed by overlap extension polymerase chain reactions (PCRs). Spytag002 gene was ordered from Addgene in pET28a-SpyTag002-MBP plasmid, and SpyCatcher002 gene was ordered in pDEST14-SpyCatcher002 plasmid. HFBI in pPICZαA was used as template for the HFBI gene. The primers for the overlap extension PCR can be found in Table S1. The removal of the Kex2 sites to make SpyTag003 and SpyCatcher003 was completed by site directed mutagenesis with primers that can be found in Table S2. The FMCscFv63-SpyCatcher003 and SpyTag003-HFBI inserts and the backbone plasmid, glyceraldehyde-3-phosphate dehydrogenase promoter (pGAPαA), were digested with XhoI and NotI restriction enzymes in NEB Buffer 3.1 at 37°C overnight. The digestions were run on a digestion gel to check the size of the DNA fragments and stop the digestion reaction. The DNA were extracted using the Qiagen Gel Extraction Kit according to manufacturer's instruction. The two fragments were ligated with the backbone plasmid overnight using T4 DNA ligase and T4 DNA ligase buffer at 16°C. The ligations were desalted using a Millipore 0.025-micron membrane filter for 1

hour. 5 μL of the desalted ligation was added to 50 μL of electrocompetent Escherichia coli strain DH5α and incubated on ice for 5 minutes. The cell and ligation mixture was then transferred to a chilled 1 mm electroporation cuvette. Using a Bio-Rad GenePulser Xcell electroporator, a 1.8 kV pulse was applied to the cell and plasmid mixture. The mixture was promptly quenched with 1 mL of low salt luria broth (LSLB). The cell and media mixture was incubated for 1 hour at 37°C with shaking. The cells were then plated on LSLB plates containing zeocin as the selectable marker. The colonies that grew on the plates were screened using colony PCR using the pGAP forward and 3AOXI primers. DNA gel electrophoresis was run for the colony PCR products to identify colonies that have an insert at the proper band size. Colonies that have the proper insert size were cultured in 1 mL of LSLB, and then the plasmids were purified using the Qiagen Miniprep Kit according to manufacturer's instruction. The purified plasmids were then sent for Sanger sequencing by GENEWIZ using the pGAP forward and 3AOXI primers to confirm the correct sequence for both FMCscFv63-SpyCatcher003 and SpyTag003-HFBI was synthesized.

Yeast transformation

The FMCscFv63-SpyCatcher003 and SpyTag003-HFBI in pGAPZαA plasmids were digested with SacI restriction enzyme and NEB Buffer 1.1 to linearize the plasmids. The linearized plasmids were ethanol precipitated and resuspend in 5 μL of nuclease free water each. Electrocompetent *Pichia pastoris* strain GS115 yeast cells were prepared using the protocol described by Sallada et al[7]. The 5 μL solution containing the linearized plasmid was added to 100 μL of the electrocompetent GS115 cells and incubated on ice for 5 minutes. The cell and plasmid mixture was then transferred to a chilled 2 mm electroporation cuvette. Using a Bio-Rad GenePulser Xcell electroporator, a 1.5 kV pulse was applied to the cell and plasmid mixture. The mixture was promptly quenched with 1 mL of chilled 1 M sorbitol. The cells recovered for 1 hour at 30°C without being shaken. 1 mL of YPD media (1% yeast extract, 2% peptone, 1% dextrose) was then added to the cells and incubated for 1 hour at 30°C with shaking. The cells were then plated on YPDS plates that contained varying concentrations (100-1000 μg/mL) of zeocin for selection. Plates were incubated at 30°C until colonies appear, around 4 or 5 days.

High Expression Colony Selection

To select the highest-expression colony for both SpyTag003-HFBI and FMC63scFv-SpyCatcher003, several colonies from each plate were selected and inoculated in 5 mL of YPG media (1% yeast extract, 2% peptone, 1% glycerol) in 50 mL conical tubes with loosened caps. The cultures were grown for three days at 28°C and shaken at 250 rpm. The tubes were centrifuged for 5 minutes to pellet the cells. 5 μL of the supernatant for each tube was applied to a nitrocellulose membrane. The membrane was blocked with a 5% dry milk/0.1% TBS-T in a Tris-buffered solution for 1 hour at room temperature. The membrane was then incubated with an HRP-conjugated rabbit anti-His tag antibody, diluted 1:1000 in the blocking solution, for 1.5 hours at room temperature. The membrane was then washed with TBS-T for 5 minutes 4 times. The membrane was then incubated for 5 minutes with Thermo Scientific SuperSignal West Dura Extended Duration substrate and imaged with the GE Amersham Imager 680. The culture that resulted in the highest-intensity spot was then used for protein production.

Protein Production and Purification

A single colony of SpyTag003-HFBI was inoculated into a 5 mL of YPG in a 50 mL conical tube with a loosened cap for gas exchange. This culture was grown at 28°C and shaken at 250 rpm until it reached saturation. The culture was spun down at 3000 rpm for 5 minutes to separate the cells, and the supernatant was discarded. The cells were then resuspended in 200 mL of YPG. The cells grew under the same conditions as before for 3 more days. Due to the surfactant nature of HFBI, the protein can be purified from both the foam and liquid fractions of the culture. For the foam fraction, 70% ethanol was sprayed into the flask to collapse the foam. The solution was collected in a 50 mL conical tube and 70% ethanol was added for a total volume at 50 mL. The solution was centrifuged at maximum speed

for 10 minutes to pellet cells and insoluble proteins. 1 M Tris pH 8 buffer was added to the supernatant for a final concentration of 50 mM to increase pH. The supernatant was ran through a 0.22 micron filter onto an immobilized metal affinity chromatography (IMAC) column to separate the protein using the His tag. After the supernatant ran through, 10 column volumes of 10 mM imidazole buffer were ran through the column. Then 4 column volumes of 500 mM imidazole buffer were ran through and collected in 1 mL aliquots. For the liquid fraction, the culture was spun down at maximum speed for 5 minutes and the supernatant was transferred to a separatory funnel with the valve closed. Triton X-114 was added with a serological pipette to a 3% volume per volume concentration to the separatory funnel. The funnel was shaken to dissolve the Triton X-114 into the culture, and the solution was incubated overnight at 37°C to allow separation. The bottom phase in the separatory funnel was collected, and isobutanol was added to the bottom phase to reach 50% of the total volume. The solution was centrifuged at maximum speed for 5 minutes. A serological pipette was used to carefully remove as much of the upper isobutanol layer as possible. The lower aqueous phase was collected with a serological pipette, carefully avoiding any leftover isobutanol layer. 1 M Tris pH 8 buffer was added to the supernatant for a final concentration of 50 mM to increase pH. The lower aqueous phase was ran through a 0.22 micron filter onto an IMAC column to separate the protein using the His tag. After the lower aqueous phase ran through, 10 column volumes of 10 mM imidazole buffer were ran through the column. 10 column volumes of 20% ethanol/50mM tris pH 8 were then added to enhance removal of residual bound Triton X-114. The column was then washed with 5 additional column volumes of 10 mM imidazole buffer. 4 column volumes of 500 mM imidazole buffer were ran through and collected in 1 mL aliquots. Samples from both the foam and liquid fractions were collected and ran on an SDS-PAGE gel to check in which samples the protein eluted. EDTA was added to the samples containing protein to eliminate any eluted nickel from the IMAC column that could cause protein precipitation. The samples were dialyzed in 1X PBS at pH 7.4. The protein concentration was quantified using the NanoDrop 1000 Spectrophotometer.

A single colony of FMC63scFv-SpyCatcher003 was inoculated directly into 50 mL of YPG in a baffled flask. This culture was grown at 28°C and shaken at 250 rpm for 3 days. The solution was centrifuged at maximum speed for 5 minutes, and the supernatant was collected. 1 M Tris pH 8 buffer was added to the supernatant for a final concentration of 50 mM to increase pH. The supernatant was ran through a 0.22 micron filter onto immobilized metal affinity chromatography (IMAC) column to separate the protein using the His tag. After the supernatant ran through, 10 column volumes of 10 mM imidazole buffer were ran through the column. Then 4 column volumes of 500 mM imidazole buffer were ran through and collected in 1 mL aliquots. The samples were ran on an SDS-PAGE gel to check in which samples the protein eluted. The samples containing protein were dialyzed in 1X PBS at pH 7.4. The protein concentration was quantified using the NanoDrop 1000 Spectrophotometer.

Protein Amidation

FMCscFv63-SpyCatcher003 and SpyTag003-HFBI were combined in a method similar to the one described in Keeble et al, 2017. Due to the low protein concentrations, the maximum available protein concentrations for FMCscFv63-SpyCatcher003 and SpyTag003-HFBI were combined instead of equal concentrations. 10 μL of each protein solution were combined in a PCR tube and incubated at room temperature for one hour, consistent with literature. Additionally, 10 μL of FMCscFv63-SpyCatcher003 was combined with 10 μL of 1X PBS at pH 7.4 and 10 μL of SpyTag003-HFBI was combined with 10 μL of 1X PBS at pH 7.4 in two separate PCR tubes. These two combinations were also incubated at room temperature for 1 hour to serve as controls. To confirm that protein amidation occurred, a protein gel was ran containing three samples: the FMCscFv63-SpyCatcher003 and SpyTag003-HFBI mixture incubated at room temperature for one hour, FMCscFv63-SpyCatcher003 alone incubated at room temperature for one hour, and SpyTag003-HFBI alone incubated at room temperature for one hour. On the protein gel, a protein band at a third

size was looked for that corresponded to the theoretical molecular weight of the combined FMCscFv63 -HFBI and was not present in the other two lanes.

Emulsion Stability Test

The protein system of FMCscFv63-HFBI and a hydrophobic colored dye, such as BODIPY, in a carrier oil, such as octane, would have been combined and sonicated on ice with a Qsonica Q125 tip sonicator in continuous mode for 10 minutes. The emulsions would then be centrifuged at 3000 rpm for 30 seconds to induce phase separation, and the bottom phase would be collected in a glass vial for stability observation. The sample would have been qualitatively observed over a period of 7 days to see if the location of the colored dye changes. The change in the emulsions would have also been tracked quantitively by looking at the droplet hydrodynamic radius with digital light scattering (DLS) with a Malvern Zetasizer Nano. 50 µL of the emulsions would have been combined with 500 µL of Milli-Q water in a DLS cuvette. The DLS autocorrection function would have been recorded at a 90-degree collection angle at 22°C. The hydrodynamic radius would have been calculated using the Zetasizer Nano Software v3.30 at day 1 and day 7.

Flow Cytometry

Confirmation of binding selectivity to the target cell type would have been done with flow cytometry. CRL-1621, CD19/CD20 positive, and CCL-155, a CD19 negative/CD38 positive, cells would have been incubated with various dilutions of the protein system. The selectivity of the protein system can be tested by using three differently colored fluorescent antibodies: anti-CD38 antibody (conjugated with a red secondary antibody), anti-CD20 antibody (conjugated with a blue secondary antibody), and the anti-His-tag antibody (conjugated with a green secondary antibody). Anti-CD38 antibody will only bind to CCL-155, anti-CD20 antibody only to CRL-1621, and the anti-His tag antibody only to the fusion protein. This labelling can be seen in Figure 1. The different colors will be used during flow cytometry to assess the populations to make sure that the cells are labeled with both blue and green, but not red and green. Additionally, the

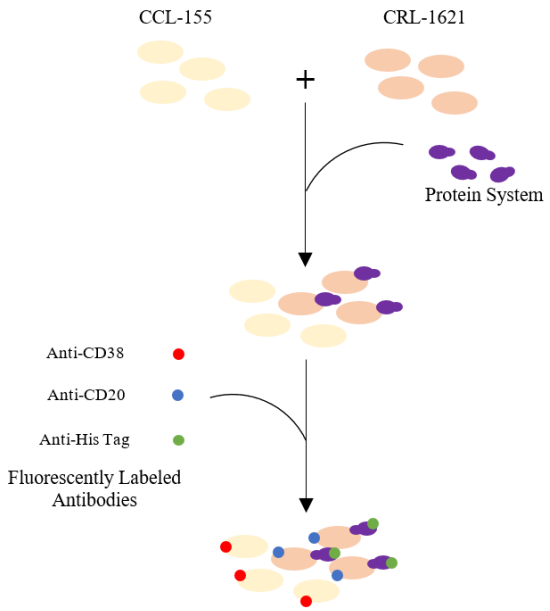

Figure 1- Fluorescent antibody labelling of CCL-155 (C19 negative, CD38 positive), CRL-1621 (CD19/CD20 positive), and FMC63scFv-HFBI to assess the binding selectivity of the FMC63scFv-HFBI to be used for flow cytometry.

binding affinity of FMC63scFv-HFBI to Daudi cells would have been found using the protocol in Nicolson et al using FMC63scFv-HFBI with the fluorescently-labeled anti-His tag antibody as the marker[8]. The flow cytometry procedures would have been done by the University of Virginia's Flow Cytometry Core.

Cell Viability Assays

Varying concentrations of the fusion protein and a hydrophobic anti-cancer drug would have been combined into emulsions via sonification. The emulsion droplets would have been incubated with CRL-1621 cells for 48 hours and subjected to the modified lactase dehydrogenase (LDH) assay protocol as described in Smith et al[14]. Cells would have been incubated with the anti-cancer drug and FMCscFv63 -HFBI emulsions. Triton X-100 would have been added to a 2% total volume to degrade the cell membranes. After centrifugation for 5 minutes at 1000 rpm, 100 µL supernatant would have been transferred to an assay plate and 100 µL reagent from an LDH assay kit would have been added. The plate would have been incubated with the reagent at room temperature for 20 minutes and imaged using a BioTek Neo2 plate reader at a wavelength of 490 nm. For a negative control, an emulsion containing no anti-cancer drug would have been

included. The protocol would have been repeated with CCL-155 cells to see the affect of the protein system on non-target cells. A negative control of the CCL-155 cells alone would have been included.

Results:

The creation of both FMC63scFv-SpyCatcher003 and SpyTag003-HFBI and ligation into the pGAPαA plasmid were confirmed with Sanger sequencing. Both proteins were successfully produced recombinantly in *Pichia pastoris* as confirmed by a Western blot. Due to COVID-19, we were unable to return to lab to complete the planned functionality experiments. The following results for the functionality experiments (emulsion stability test, flow cytometry, and cell viability assays) are what one would expect to get from performing these experiments based on what was found in literature.

Protein Amidation Confirmation

When protein amidation occurs, the resulting protein's molecular weight would equal the sum of the two individual proteins. To check the molecular weights, we ran a protein gel as seen in Figure 2. We predicted that the lane containing FMC63scFv-HFBI would also contain bands for FMC63scFv-SpyCatcher003 and SpyTag003-HFBI as all the protein may not link during the 1 hour incubation period. The predicted protein gel can be seen in Figure 2A. The experimental protein gel, seen in Figure 2B, is consistent with

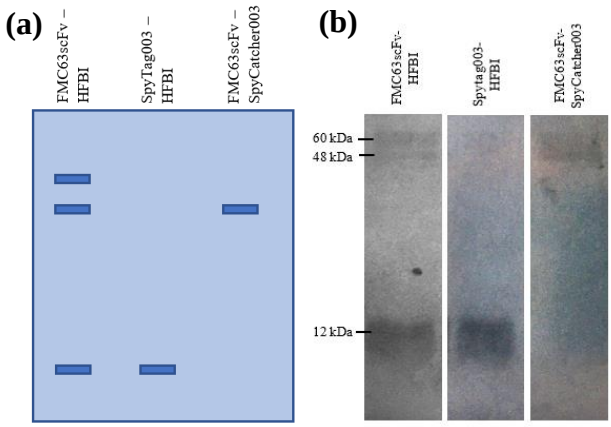

Figure 2 – Protein gels to confirm linking of FMC63scFv-SpyCatcher003 and SpyTag003-HFBI. (a) Theoretical protein gel confirming protein amidation in lane 1. (b) Experimental protein gel confirming protein amidation in lane 1. Wells of SpyTag003-HFBI alone and FMC63scFv-SpyCatcher003 alone were ran as controls.

the predicted results. In the first lane containing the sample from the FMC63scFv-SpyCatcher003 and SpyTag003-HFBI mixed incubations shows three bands corresponding to FMC63scFv-HFBI, FMC63scFv-SpyCatcher003, and SpyTag003-HFBI. However, the bands on this gel, especially at the higher molecular weights, are very faint. This is consistent with lower protein concentration measured for FMC6scFv-SpyCatcher003 in the dialyzed aliquots.

Emulsion Stability Test

Emulsion stability results can be predicted off of previous literature. Sallada et al ran emulsion stability tests for wildtype HFBI and obtained the results seen in Figure 3[7]. Figure 3A and 3B show

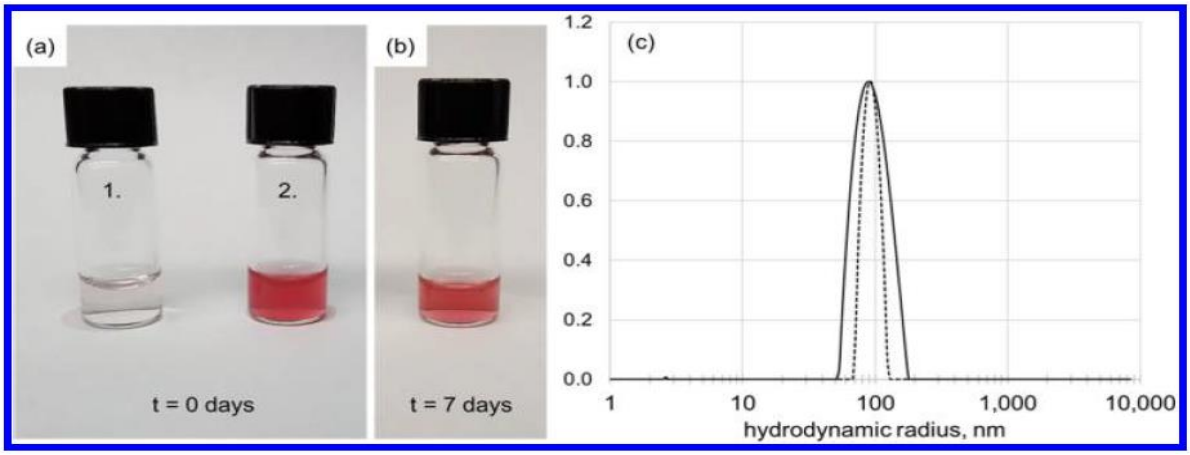

Figure 3- Hydrophobin-stabilized Oil Red O-stained octane emulsions. (a)Sample 1: t = 0 days, 10 µM IAM/+DTT hydrophobin bottom phase. Sample 2: t = 0 days, 10 µM IAM/−DTT hydrophobin bottom phase. (b) IAM/−DTT hydrophobin bottom phase at 7 days. (c) Dynamic light scattering hydrodynamic radius intensity-weighted distribution of the IAM/−DTT hydrophobin emulsion at 0 days (- - -) and 7 days (---). Figure and caption taken from: Sallada, Dunn, and Berger. *Biochemistry*, **2018** *57*(5), 645-653.

how the emulsion stability can be qualitative tracked to see if there is a difference between day 1 and day 7 such as phase separation. Figure 3C shows the DLS data for wildtype HFBI at day 1 and day 7. The emulsions had a hydrodynamic radius of 96.43 nm and 92.77 nm at days 1 and 7, respectively[7]. We predict that the FMC63scFv-HFBI would behave similarly to wildtype HFBI and remain stable over 7 days. To compare the protein system emulsion stability over time, we would have done a paired two tail t-test of the hydrodynamic radius from DLS data of the emulsions at Day 1 and Day 7 to see if there is a significant ($p < 0.05$) difference in size. Due to the larger-sized protein (HFBI linked to FMCscFv versus HFBI alone) making up the emulsion, we expect a slightly larger hydrodynamic radius than the wildtype HFBI.

Flow Cytometry

Had the flow cytometry experiments actually been performed, the flow cytometry software would have created a population dot plot. The dot plot would have been analyzed to make sure that there are two populations: one labeled with green and blue fluorescent markers and one labeled only with red fluorescent markers, following the labeling scheme discussed in the methods section. If there were two distinct populations with one labeled blue and green and one labeled red, that would show that the protein system is only targeting cell lines that are CD19 positive. However, if there was green labeling in with the red fluorescent labeling, that would show that the protein system is not targeting CD19 exclusively as it is binding with a CD19 negative cell line.

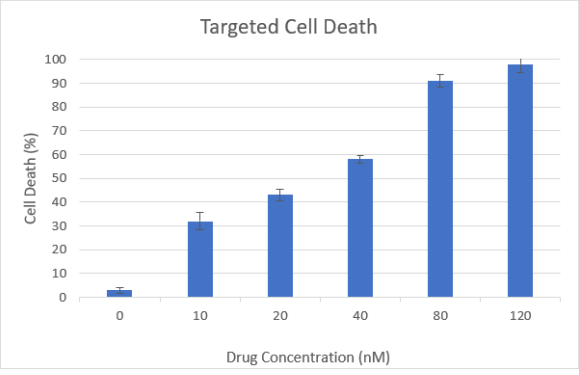

Figure 5 - Cell death percentages found from an LDH assay. Several assays were performed at various concentrations to determine the minimum concentration of drug for significant cell death.

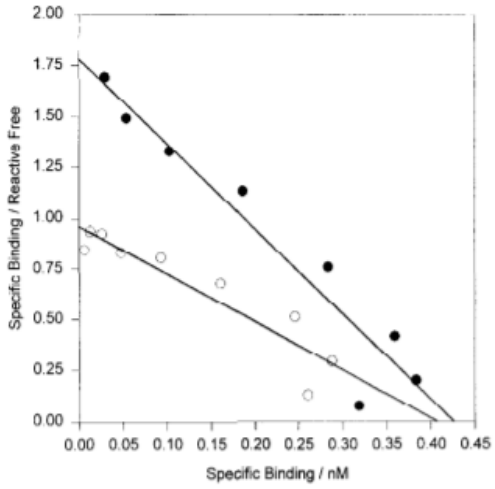

Figure 4- Scatchard plot for the calculation of binding affinity of FMC63scFv (black circles) to CD19 positive Daudi cells. K_a was found to be 4.2×10^9 M^{-1}.

Figure taken from: Nicholson, Lenton, Little, Decorso, Lee, Scott, Zola, Hohmann. *Molecular Immunology*, **1997** 34 (16), 1157-1165.

Furthermore, the binding affinity of the FMC63scFv-HFBI would have been quantified using a Scatchard plot like the one seen in Figure 4, calculated following a similar method as Nicholson et al[8]. In Nicholson et al, a binding affinity of 4.2×10^9 M^{-1} was found for FMC63scFv to Daudi cells[8]. Using our calculated binding affinity, we would have performed an unpaired one-tailed t-test between the binding affinity values to determine if the linkage to HFBI had a significant ($p < 0.05$) effect on binding affinity of the FMC63scFv.

Cell Viability Assays

Had the cell viability assays for targeted cells been performed, the results could help indicate the dosage of anti-cancer drug required for cell death. Figure 5 shows example data that would have been collected from the LDH cell viability assay. The concentrations and cell death percentages are loosely based on Milano et al's study on doxorubicin, a hydrophobic anti-cancer drug that could be used in the protein system for B-ALL treatment[15]. An unpaired one-tailed t-test between the lowest anti-cancer drug concentration (10 nM) and the no drug control would show if there is significant ($p < 0.05$) cell death with the addition of an anti-cancer drug. This would show that the drug is entering the cell to cause cell death. Higher concentrations of drug

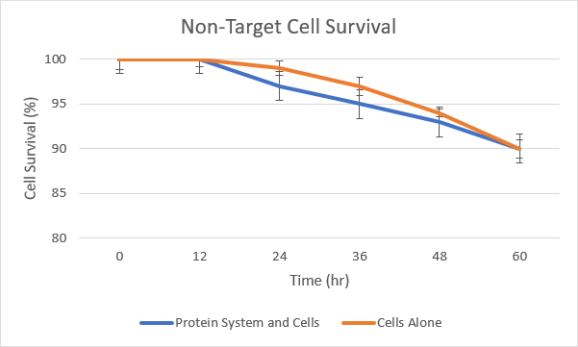

Figure 6 – Cell survival percentages found from an LDH assay. Two trials were performed, one with the protein system without drug and the other with cells alone as the negative control.

could also be investigated to increase the cell death percentage.

Had the cell viability assays for non-targeted cells been performed, the results could help indicate the toxicity of the protein system. Figure 6 shows example data that would have been collected from the LDH cell viability assay. Data would have been collected at various time points for the cells alone and the cells mixed with the protein system. An unpaired one-tailed t-test between the cells with the protein system and the no protein control cells would be performed at the various time points to determine if there was significantly ($p < 0.05$) more cell death in the cells containing the protein system over the cells alone. If there is not significantly more cell death when the protein system is present, it can be concluded that the protein system is not toxic to non-target cells.

Discussion:

Preliminary results from the protein gel suggest that the proteins can be linked via the SpyTag-SpyCatcher mechanism, but further experiments need to be performed. Higher concentrations of the protein system need to be ran on the protein gel or a more sensitive stain, such as silver stain, needs to be used to obtain clearer bands. Additionally, the protein gel needs to be ran at a lower voltage for a longer time to obtain better separation at higher molecular weights to be able to better differentiate between FMCscFv63-SpyCatcher003 and FMCscFv63-HFBI. However, no conclusions can be drawn on the possibility of this protein system as a drug delivery mechanism for B-ALL as the functionality experiments (emulsion stability test, flow cytometry, and cell viability assays) were unable to be performed because we were not able to return to lab due to COVID-19.

If the results of the functionality test suggest that the protein is not suitable as a drug delivery mechanism for B-ALL, it would be informative to see where the protein system failed. If the emulsion stability test lead to inconclusive results, there are other tests to assay surface activity of the protein system like water contact angle (WCA) tests to assess surface activity or Förster Resonance Energy Transfer (FRET) to assess the protein-protein interactions in the self-assembly. Both WCA tests and FRET have been used in the assessment of HFBI's ability to self-assemble[7]. If the results from the emulsion stability test suggest that the protein system does not self-assemble, mixed micelles of the protein system and wildtype HFBI can be made. The wildtype HFBI will aid in the self-assembly of the micelle while the protein system retains the targeting capability. If the results of the flow cytometry test are inconclusive, there are other assays to look at the binding affinity of the protein system. A competitive ELISA can be used as described in Yao et al[16]. If the results show that the protein system is not selectively targeting CD19 positive cell lines, the linker connecting the FMC63scFv and the SpyCatcher003 protein can be modified so that the SpyCatcher003 is not interfering with the targeting ability of the FMC63scFv. If the results from the LDH assays are inconclusive, there are a variety of other cell viability and toxicity assays. An MTT assay, luminescence ATP assay, or a red/green cell death assay can all be performed. If the LDH cell viability results for targeted cells show that CD19 positive cells are not dying, the concentration of anti-cancer drug in the protein system emulsion can be increased. However, the issue may be that the protein-drug is not entering the cell, so the drug is not being released to kill the cell. To combat that issue, the emulsions can be made with a mix of FMC63scFv-HFBI and transferrin-HFBI fusion proteins. The inclusion of transferrin has been shown to increase uptake of the emulsions into the cell[17]. If the LDH cell viability results for non-targeted cells show that the protein system alone is causing cell death, the concentration of the protein system can be lowered to reduce the effective dose.

If the results of the functionality tests did suggest that the protein system kills targeted cells but not non-targeted cells, further characterization and optimization of the protein system could be done to increase efficacy. The protein system would then need to be tested in an *in vivo* setting to obtain more data about the protein system as a drug delivery mechanism for B-ALL. With positive results, this project would offer a novel mechanism to deliver hydrophobic anti-cancer drugs to lymphoid cells for the treatment of B-ALL. This mechanism is targeted to the lymphoid cells and the drug is released within the cell, so it is not systemic like chemotherapy and will not affect the surrounding tissue. This mechanism would not have to be delivered invasively which will allow for easier delivery. Furthermore, this would eliminate the issue of the location of the cancerous cells in the case of metastasis. This protein-based drug delivery system would also provide a cheaper alternative to CAR-T cell therapy. On average in the United States, biologics cost $45 per day[18]. While this is higher than the cost of a small molecule drug, it is significantly less than the cost of CAR-T cell therapy[18]. For $45 per day, one could afford a biologic treatment every day for almost 29 years for the price of a singular treatment of CAR-T cell therapy. Additionally, hydrophobins are non-immunogenic, which offers an advantage over the immunogenic chemical surfactants that are typically used in cancer drug delivery[4].

In this proposed method, any hydrophobic anti-cancer drug can be used in the self-assembled HFBI structure. If the patient is not responding well to a certain anti-cancer drug or is unable to use a specific anti-cancer drug, another hydrophobic anti-cancer drug can be easily added into the self-assembled HFBI structure. Switching out the anti-cancer drug can also be used if the cells are becoming desensitized to the current drug that is being used in the treatment. Additionally, this project will create a novel targeted drug delivery mechanism that can be applied to multiple types of cancer. CD19 can be used as a target for other cancers such as B cell non-Hodgkin's Lymphoma and chronic lymphoma leukemia[19]. Furthermore, the SpyCatcher003 protein can be linked to other targeting peptides or scFvs while the SpyTag003-HFBI can remain unchanged. Epidermal growth factor receptor (EGFR)-binding peptide is a peptide sequence that has been developed and optimized to target EGFR with limited off-target effects[20]. EGFR is often overexpressed in many different cancer cell lines, such as esophageal, breast, and prostate cancers, and previous literature has shown the ability of EGFR-binding peptide to target these cancer types[21]. Therefore, these cell lines and therefore the associated cancers can also be targeted with the EGFR binding peptide-HFBI protein system in a similar method to the FMC63scFv-HFBI.

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References:

1. Jabbour, E., O'Brien, S., Konopleva, M. & Kantarjian, H. New insights into the pathophysiology and therapy of adult acute lymphoblastic leukemia. *Cancer* **121**, 2517–2528 (2015).

2. Terwilliger, T. & Abdul-Hay, M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer J.* **7**, 577 (2017).

3. Cooper, S. L. & Brown, P. A. Treatment of Pediatric Acute Lymphoblastic Leukemia. *Pediatr. Clin. North Am.* **62**, 61–73 (2015).

4. Berger, B. W. & Sallada, N. D. Hydrophobins: multifunctional biosurfactants for interface engineering. *J. Biol. Eng.* **13**, 10–17 (2019).

5. Prasad, V. Tisagenlecleucel — the first approved CAR-T-cell therapy: implications for payers and policy makers. *Nat. Rev. Clin. Oncol.* **15**, 11–12 (2018).

6. Aimanianda, V. *et al.* Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* **460**, 1117–1121 (2009).

7. Sallada, N. D., Dunn, K. J. & Berger, B. W. A Structural and Functional Role for

Disulfide Bonds in a Class II Hydrophobin. *Biochemistry* **57**, 645–653 (2018).

8. Nicholson, I. C. *et al.* Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma. *Mol. Immunol.* **34**, 1157–1165 (1997).

9. Kochenderfer, J. N. *et al.* Construction and Pre-clinical Evaluation of an Anti-CD19 Chimeric Antigen Receptor. *J. Immunother.* **32**, 689–702 (2009).

10. Nelson, A. L. Antibody fragments. *mAbs* **2**, 77–83 (2010).

11. Zakeri, B. *et al.* Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc. Natl. Acad. Sci.* **109**, E690–E697 (2012).

12. Keeble, A. H. *et al.* Evolving Accelerated Amidation by SpyTag/SpyCatcher to Analyze Membrane Dynamics. *Angew. Chem. Int. Ed.* **56**, 16521–16525 (2017).

13. Sallada, N. D., Harkins, L. E. & Berger, B. W. Effect of gene copy number and chaperone coexpression on recombinant hydrophobin HFBI biosurfactant production in Pichia pastoris. *Biotechnol. Bioeng.* **116**, 2029–2040 (2019).

14. Smith, S. M., Wunder, M. B., Norris, D. A. & Shellman, Y. G. A Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. *PLOS ONE* **6**, e26908 (2011).

15. Milano, G. *et al.* Doxorubicin weekly low dose administration: in vitro cytotoxicity generated by the typical pharmacokinetic profile. *Eur. J. Cancer* **28**, 1881–1885 (1992).

16. Yao, G. *et al.* Identification of core functional region of murine IL-4 using peptide phage display and molecular modeling. *Int. Immunol.* **18**, 19–29 (2006).

17. Reuter, L. J. *et al.* Coating Nanoparticles with Plant-Produced Transferrin–Hydrophobin Fusion Protein Enhances Their Uptake in Cancer Cells. *Bioconjug. Chem.* **28**, 1639–1648 (2017).

18. Blackstone, E. A. & Joseph, P. F. The Economics of Biosimilars. *Am. Health Drug Benefits* **6**, 469–478 (2013).

19. Park, J. H., Geyer, M. B. & Brentjens, R. J. CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date. *Blood* **127**, 3312–3320 (2016).

20. Kikuchi, O. *et al.* Novel EGFR-targeted strategy with hybrid peptide against oesophageal squamous cell carcinoma. *Sci. Rep.* **6**, 1–12 (2016).

21. Kohno, M. *et al.* A novel hybrid peptide targeting EGFR-expressing cancers. *Eur. J. Cancer* **47**, 773–783 (2011).

Supplemental Materials:

FMC63scFv-SpyCatcher003

SDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHS
GVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGSTSGS
GKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRK
GLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYY
YGGSYAMDYWGQGTSVTVSGGGGSGGGGSGGGGSMVTTLSGLSGEQGPSGDM
TTEEDSATHIKFSKKDEDGRELAGATMELRDSSGKTISTWISDGHVKDFYLYPGKY
TFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHTGSSGSAAASFLEQKLI
SEEDLNSAVDHHHHHH

SpyTag003-HFBI

VPTIVMVDAYKPYKGSGESGSNGNGNVCPPGLWSNPQCCATQVLGLIGLDCKVPS
QNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGAHHHHHH

Figure S1- Amino acid sequences of FMC63scFv-SpyCatcher003 and SpyTag003-HFBI proteins. Yellow is FMC63scFv, blue is SpyTag003, red is linkers, purple is SpyCatcher003, green is the HFBI, and orange is the His tags.

Table S1- Overlap Extension PCR Primers for FMC63scFv-SpyCatcher002 and SpyTag002-HFBI

Primer Name	Primer Sequence
spycatcher_GGGS_f	GGAGGTGGTGGATCCATGGTAACCACCTTA
spycatcher_Noti_R	AAAAAAGCGGCCGCGCTACCACTGGATC
FMC63scfv_xhoi_f	GTATCTCTCGAGAAAAGAGAGGCTGAAGCTTCCGAC
FMC63scfv_GGGS_r	GGATCCACCACCTCC
HFBI_chis6_noti_R	AAAAAAGCGGCCGCTTAGTGATGGTGGTGATGATGTGCTCCAACTGCAGTCTG
HFBI_spytag_F	GTAGTGGTGAAAGTGGTTCCAACGGTAACGGAAAT
spytag_xhoi_F	GTATCTCTCGAGAAAAGAGAGGCTGAAGCTGTGCCTACTATCGTGATGGTGGACGCC
spytag_R	ACCACTTTCACCACTAC

Table S2- Mutagenesis Primers to Remove Kex2 Sites in SpyCatcher002 and SpyTag002

Primer Name	Primer Sequence
spycatcher002_R33K_F	ACCCATATTAAATTCTCAAAAAAGGATGAGGACGGCCGTGAGTTA
spycatcher002_R33K_R	TAACTCACGGCCGTCCTCATCCTTTTTTGAGAATTTAATATGGGT
spytag002_R12P_f	GGACGCCTACAAGCCTTACAAGGGTAGTG
spytag002_R12P_r	CACTACCCTTGTAAGGCTTGTAGGCGTCC