Iran Agricultural Research (2025) 44 (2) 88-96.

Shiraz University

Iran Agricultural Research

Journal homepage: https://iar.shirazu.ac.ir/?lang=en

Research Article

In planta transient recombinant production of a bispecific T-cell engager: Blinatumomab

Saeideh Dianatkhah[a], Chaitra Hiremath[b], Stanislav Melnik[b], Lin Sun[b], Amin Ramezani[c,d], Ali Niazi[a], Alireza Afsharifar[e], Ali Moghadam[a]*, Somanath Kallolimath[b]*

[a] Institute of Biotechnology, Shiraz University, Shiraz, I. R. Iran
[b] Institute of Plant Biotechnology and Cell Biology, Department of Biotechnology and Food Sciences, BOKU University, Vienna, Austria
[c] Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, I. R. Iran
[d] Department of Medical Biotechnology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, I. R. Iran
[e] Research Center of Plant Virology, School of Agriculture, Shiraz University, Shiraz, I. R Iran

ARTICLE INFO

Keywords:
Antibody
Blinatumomab
CD19
CD3

Received: 12 June 2025
Revised: 13 August 2025
Accepted: 16 August 2025

ABSTRACT- Bispecific T-cell engagers (BiTEs) are increasingly being recognized as promising therapeutic molecules in tumor management. However, these largely artificial and complex antibody-based molecules are often difficult to express in conventional platforms. Here, we evaluated the recombinant expression of the approved dual-targeting bispecific T-cell engager Blinatumomab (BLIN) for its production in plants. A highly potent viral vector carrying the coding sequence of BLIN with a His tag was used for transiently expressing Blinatumomab in *Nicotiana benthamiana*. Four days post-DNA construct delivery, immunoblotting exhibited BLIN-specific signals in total soluble proteins (TSP) as well as in the intercellular fluid (IF), indicating secretion of the recombinant protein to the extracellular space. Co-expression of an RNA silencing suppressor (p19) increased BLIN-specific signals. While Ni-NTA purification from TSP resulted in insufficient BLIN quality, IF-derived BLIN was eluted as a single band at the correct molecular weight. The yield of IF-BLIN was approximately 15 µg/g fresh leaf weight, which is ten times lower than usually obtained for monoclonal antibodies (mAbs). Chromatographic profiling of IF-BLIN exhibited significant amounts of unwanted high molecular weight forms next to the targeted monomeric fraction. After purification of this fraction, the functional activity of IF-BLIN was confirmed by binding to cells expressing the targeted antigens CD3 or CD19. Collectively, we showed the successful production of functionally active BLIN in plants. Further optimization steps are required to improve the yield and quality of plant-produced BLIN.

INTRODUCTION

Bispecific T-cell engagers (BiTEs) in biology usually act as immunotherapeutic molecules intended for the treatment of cancer. Blinatumomab (BLIN), is the first approved BiTE for use in B-cell acute lymphoblastic leukemia (Sanford, 2015; Burt et al., 2019). In more than 90% of acute lymphoblastic leukemia cases, the CD19 receptor, a B-cell-specific surface antigen, is expressed on the surface of leukemia cells (Stanciu-Herrera et al., 2008). This receptor acts as a potential target in B-cell malignancy and autoimmune diseases. BLIN is an antibody fragment that consists of two different single-chain variable fragments (ScFv). These two arms are joined by a glycine-serine linker, which is specific for CD19 and CD3, respectively. It carries the N-terminal anti-CD19 variable fragment derived from murine mAb HD37 and the C-terminal anti-CD3 binding variable fragment derived from murine mAb L2K-07 (Moreau et al., 2022).

Its mechanism of action involves binding to CD19, expressed on malignant B-cells, and recruitment of T-cells by binding to CD3 at the same time. Such structure and specificity allow a BiTE to physically bring a T-cell toward a tumor cell in proximity, which ultimately stimulates T-cell activation, tumor killing, and cytokine production (Wu et al., 2015).

*Corresponding Authors: Associate Professor, Institute of Biotechnology, Shiraz University, 71441-65186 Shiraz, I. R. Iran; Professor, Institute of Plant Biotechnology and Cell Biology, Department of Biotechnology and Food Sciences, BOKU University, 1190 Vienna, Austria
E-mail address: ali.moghadam@shirazu.ac.ir; somanath.kallolimath@boku.ac.at
DOI: 10.22099/iar.2025.53455.1696

Recently, five more BiTE molecules, i.e., Teclistamab, Elranatamab, Talquetamab, Glofitamab, and Epcoritamab have been approved by the FDA and are applied for the treatment of cancers such as multiple myeloma, leukemia, and lymphoma (Moreau et al., 2022; Keam, 2023; Hutchings et al., 2021; Thieblemont et al., 2023; Dhillon, 2023).

The recombinant production of BiTEs poses a greater challenge regarding quantity, quality, and stability compared with full monoclonal antibodies. ScFvs are often plagued by too low expression, inefficient purification, and aggregation, hindering the clinical investigation of such molecules (Pirkalkhoran et al., 2023). Despite clinical efficacy, BiTEs also have disadvantages such as fast serum clearance, lack of effector functions like antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement activation, due to the absence of crystallizable fragment (Fc) (Wang et al., 2019; Thoreau et al., 2022; Pirkalkhoran et al., 2023).

BLIN does not need extensive post-translational modifications such as glycosylation. Thus, non-mammalian expression systems were also investigated. A comparative study on the expression and functional activity of BLIN produced in *Escherichia coli (E. coli)* and CHO cells exhibited higher expression levels in the bacterial system, at the cost of lower binding efficiency compared to counterparts produced in the CHO cell line. In a bacterial expression system, a protein often ends up in inclusion bodies, making it challenging to isolate the fully functional protein (Naddafi et al., 2018; Maghsoodi et al., 2023).

Plants are broadly recognized as a potent alternative platform to produce recombinant proteins. In recent decades, significant advances have been made in producing transient expression vectors that allow rapid production of recombinant proteins within days after infiltration of DNA constructs into leaves (Eidenberger et al., 2022; Coates et al., 2022).

For the production of glycoproteins carrying humanized glycans, advanced tools that allow the expression of proteins with targeted human-type N-glycans and large homogeneity have been established (Montero-Morales & Steinkellner, 2018). Production of antibody isotypes and virus-like particles (VLPs) has provided strong evidence that plants can efficiently produce fully assembled functional proteins at mg levels per kg of fresh leaf material (Eidenberger et al., 2023; Balieu et al., 2022; Asghar et al., 2022).

Due to the increased clinical efficacy of artificially designed therapeutic molecules like bispecific antibodies and ScFvs, attempts were made to produce these molecules using plant-based transient expression modules. Multiple targets are envisaged, e.g., anti-cancer, viral, and toxic molecules (Ravanrouy et al., 2021; Amirzadeh et al., 2023; Galeffi et al., 2005; Gomes et al., 2019; Seber Kasinger et al., 2019; Phoolcharoen et al., 2017; Lai et al., 2014; Bulaon et al., 2024). In general, ScFvs suffer from low expression levels and instability.

This study focuses on the investigation of BLIN antibody expression and purification in the *Nicotiana benthamiana* (*N. benthamiana*) plant expression system.

MATERIALS AND METHODS

Plant expression host

As plant host, to express recombinant proteins, the glycoengineered *N. benthamiana* (ΔXTFT) plants (Strasser et al., 2008) with down-regulation of plant-specific glycan epitopes (core α1,3-fucose and β1,2-xylose) were served. The plants were grown under controlled conditions at 24 degrees Celsius, 60% relative humidity, and 16/8 h light/dark photoperiod in a growth chamber.

Viral-based vector for transient expression of Blinatumomab

A synthetic cDNA fragment (1515 bp) corresponding to the blinatumomab with poly-Histidine tag was codon optimized for *N. benthamiana* with flanking BsaI sites and cloned into a BsaI-digested viral-based magnICON® vector containing α-amylase signal peptide for secretion (pICH26211α) (Marillonnet et al., 2005). Recombinant *Agrobacterium tumefaciens* strain GV3101 (pMP90) was transformed using the derived expression vector pICH26211α-Blinatumomab (Fig. 1A).

The strain including the confirmed vector carrying Blinatumomab construct was applied for the succeeding agroinfiltration experiments. Sequence information was presented in supplementary (Fig. 1B).

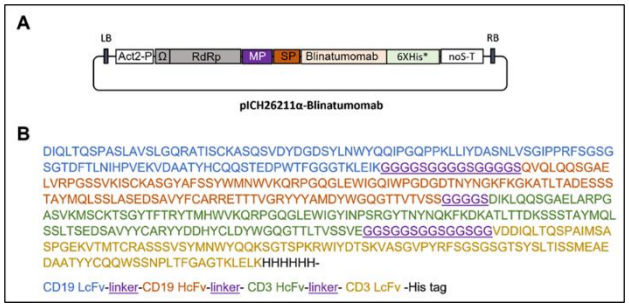

Fig. 1. The expression vector of pICH26211α-Blinatumomab for transient expression of Binatumomab in *Nicotiana benthamiana*. (A) The final recombinant Binatumomab gene construct. Abbreviations are as below: Act2-P, *Arabidobsis. thaliana* actin 2 gene promoter; TVCV genetic elements (Ω, omega leader; RdRP, RNA-dependent RNA polymerase); MP, movement protein; SP, barley α-amylase signal peptide; Blinatumomab CDS with 6XHis tag, stop codon (*); nos-T, nopaline synthase terminator. RB and LB, right and left T-DNA borders, respectively; (B) Blinatumomab amino acid sequence details; glycine-serine linker was underlined.

In planta expression of Blinatumomab

Blinatumomab were extracted according to published protocols (Kallolimath et al., 2024; Castilho at al., 2010). In brief, *Agrobacterium* strains separately carrying blinatumomab and p19 viral suppressor genes were used to express recombinant protein. These strains were grown overnight, centrifuged, and subsequently resuspended in infiltration buffer. Optical density was assessed at 600 nm (OD_{600}). The final OD_{600} was set to 0.1 and 0.2 for blinatumomab- and p19-carrying bacterial cells, respectively. Using a syringe, agrobacterial suspensions were delivered to leaves of four- to five-week-old plants. On day four after infiltration (dpi) the infiltrated leaves were harvested to extract total soluble protein (TSP) and the sample was used to determine BLIN expression.

The intercellular (apoplastic) fluid (IF) was collected by submerging intact infiltrated leaves in a beaker containing IF extraction buffer (100 mM TrisHCl, 10 mM $MgCl_2$, 2 mM EDTA; pH 7.5).

Then, the leaves were vacuum infiltrated with the IF buffer. The excess liquid on the surface of the infiltrated leaves was removed by pressing between paper towels. To avoid the leaves coming in contact with the collected IF, the infiltrated leaves were placed in centrifuge cups with cloth mesh. To release the IF from the leaves, the tubes were centrifuged at 800 × *g* for 5 min at 4 °C. The method was described previously in the literature (Keshvari et al., 2024).

Ten µL of infiltrated leaf extract or 10 µg of total soluble protein (TSP) of BLIN was separated on 12% SDS-PAGE, followed by immunoblotting with anti-6xHis-tag antibody (Invitrogen MA1-135) at 1:2000 dilution to determine the expression of the antibody. A secondary antibody anti-mouse IgG-HRP (Promega W4021) at 1:10000 was used for detection.

Purification of Blinatumomab

Recombinant blinatumomab expressed in the plant's leaves was purified by affinity chromatography using Ni-NTA beads (QIAGEN). About 25 mL of intercellular fluid isolated from ~60 g of leaf tissue was used for purification. The extracted IF from leaves was loaded onto a manually packed column, which was pre-equilibrated with 10 column volumes (CV) of binding buffer containing 50 mM Na_2HPO_4, 300 mM NaCl, 10 mM Imidazole, at the flow rate of 1.5 mL/min. The column was washed with 20 CV of washing buffer, which contained 50 mM Na_2HPO_4, 300 mM NaCl, and 20 mM Imidazole. Elution of BLIN was done in 1 mL fractions of an elution buffer containing 50 mM Na_2HPO_4, 300 mM NaCl, and 250 mM Imidazole, and then eluates were dialyzed overnight against 1x PBS. After dialysis of the affinity-purified protein, the samples were concentrated. The protein was studied more by size-exclusion chromatography. Purified BLIN sample (100 µg) was injected into a Superdex 200 column (10 mm × 300 mm, Cytiva) connected to an HPLC Prominence LC20 system (Shimadzu). Runs were conducted at a flow rate of 0.75 mL/min at 25 °C in PBS with 200 mM NaCl (pH 7.4). The molecular mass was assessed by a MALS Heleos Dawn8C plus QELS detector (Wyatt Technology, USA). Monomeric fractions were collected and stored at -80 °C until further use. The analytical SEC run was performed similarly by injecting only 10 µg of protein.

Peptide bapping

LC-MS/MS analysis was performed as previously described (Safonov et al., 2021). Briefly, the purified BLIN protein was excised from an SDS-PAA gel and then was S-alkylated with iodoacetamide and digested with Trypsin (Promega). The digested samples were loaded onto a nanoEase C18 column. Orbitrap MS (Thermo, Exploris 480) equipped with the standard H-ESI source in positive ion, DDA mode (= switching to MSMS mode for eluting peaks) was used for detection. Samples were analyzed with an LC-ESI-MS system (Thermo Orbitrap Exploris 480, Thermo Scientific). For peptide mapping the files were analyzed using PEAKS (Bio-informatics Solutions Inc, Waterloo, Ontario, Canada), which is suitable for performing MS/MS ion searches.

CD19 and CD3 binding assay

Cell culture

Cell culture and binding assay was performed based on previously reported protocols (Seigner et al., 2023; Zajc et al., 2022). Three cell lines, Nalm6, Raji, Jurkat, and A549 cells (all from ATCC), were cultured in complete medium. Every two to four days, cells were passaged by counting and diluting in a fresh complete medium. Cell counting was performed by dead-live staining using Trypan blue (Sigma-Aldrich) and the TC20 Automated Cell counter (Bio-Rad).

Titration of Blinatumomab on Nalm6 Cells CD19-positive Nalm6 cells (stably expressing GFP) were spiked into CD19-negative A549 cells to avoid ligand depletion. Cells were counted and washed. The plate was centrifuged and the supernatant was discarded before the addition of either BLIN or FMC63 (CD19-targeting ScFv) control (both containing a His-tag) at the indicated concentrations (ranging from 0.05 nM to 100 nM). A staining buffer was added to the negative control. Cells were incubated for 4 h at 4 °C while shaking (180 rpm). After this incubation, cells were washed once, the supernatant was separated and 200 µL of cold staining buffer was added. The second centrifugation step was done under the same condition. After centrifugation, the supernatant was removed. Secondary staining was performed by the adding 25 µL anti-Penta His antibody-AF647 (1:40 dilution, QIAGEN). Before the last washing step was conducted as described above, cells were again incubated for 30 min at 4 °C on a shaker under shaking conditions. The cell pellets derived from previous steps were immediately resuspended in the staining buffer before the measurement. Data was acquired on a Cytoflex S 4 Laser device (Beckman Coulter). Analysis was conducted using gating on GFP-positive Nalm6 cells and evaluating the binding signal of anti-Penta His-AF647.

Geometric mean fluorescence intensities of binding were background-subtracted and a 1:1 binding model was fitted to the data, as previously described, to calculate K_D values (Zajc et al., 2022).

In vitro CD3 and CD19 binding assay

To test the binding capacity of BLIN to CD3 and CD19, Jurkat cells expressing CD3 and Raji cells expressing high levels of CD19 were used. Thus, 5×10^4 cells were seeded in a 96-well V-bottom plate, 100 nM BLIN was added and incubated for 4 h at 4 °C while shaking (180 rpm). Secondary staining was performed with anti-Penta His-AF647. Data was acquired on a Cytoflex S 4 Laser device (Beckman Coulter). Analysis was performed by gating on GFP-positive Jurkat or Raji cells.

RESULTS AND DISCUSSION

Expression of Blinatumomab

The viral expression vector carrying the respective coding sequence (pICH26211α-Blinatumomab, Fig. 1A), was delivered through agroinfiltration to N. benthamiana (ΔXTFT) glycosylation mutant lacking plant-specific fucosyltransferase and xylosyltransferase (Strasser et al., 2008) to generate BLIN in this line. Four days post-infiltration, recombinant protein expression was observed in IF, indicating proteins secreted to apoplast, as well in TSP by Western blotting. A signal at ~55 kDa which corresponds with the size of the full-length BLIN, was detected by anti-His immunoblotting of TSP and IF samples under reducing conditions. The signal was intensified by co-expressing BLIN with p19. No degradation products were detected in IF samples, but TSP showed additional faint signals between 45 kDa and 35 kDa (Fig. 2A) indicating degradation products.

Purification of plant-derived Blinatumomab

The recombinant BLIN (IF-BLIN) eluate monitored by SDS-PAGE under reducing conditions appeared as a single band at 55 kDa position (Fig. 2B). No aggregation or degradation products were visible, and IF-BLIN yield was 15 µg/g fresh leaf weight. Since ScFvs and other recombinant proteins which expressed in plants and bacterial expression platforms tend to form aggregates and undergo degradation (Wang et al., 2019; Liu et al., 2022; Pirkalkhoran et al., 2023), IF-BLIN was subjected to size-exclusion chromatography (SEC). As a result, the SEC analysis exhibited two major peaks assigned as aggregates (A) and monomers (M), and a small peak corresponding to degradation products. The three fractions were separately collected in (Fig. 2C). Evidently, aggregates accounted for approximately 70% of the purified product. Analytical HPLC was used to further assign fractions A and M for aggregates and monomers, respectively (Fig. 3). The figure shows a monomer form of Blinatumomab and aggregates form in the purified sample.

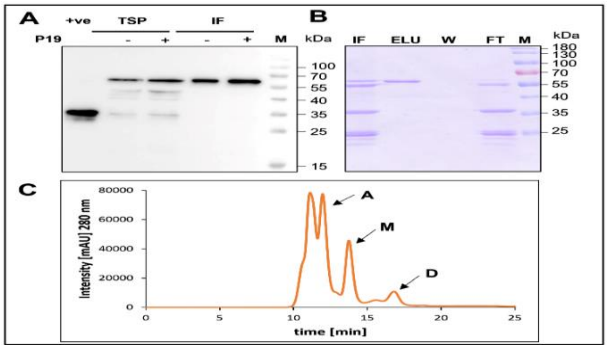

Fig. 2. Characterization of plant-derived Binatumomab (A) Anti-His immunoblot of total soluble proteins (TSP) and intercellular fluid (IF) extracted from leaf tissue infiltrated with pICH26211α-Blinatumomab with (+) or without (-) p19, +ve showed purified protein with His-tag as control. (B) SDS-PAA gel for purified Blinatumomab. Abbreviations are as below: IF, Intercellular fluid; ELU, purified elution; w, wash, and FT, flow through in reduced condition. (C) SEC profile of purified Intercellular fluid of Blinatumomab aggregates. Abbreviations are: A, aggregates; M, monomer; D, degradation products.

These results confirm the successful purification of blinatumomab and provide an accurate representation of the distribution of monomeric and aggregated forms. The analysis of these fractions will be crucial for evaluating the structural integrity and functionality of the purified protein.

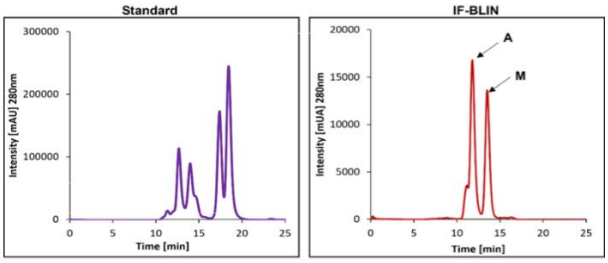

Fig. 3. Analytical chromatogram obtained by HPLC of Ni-NTA-purified recombinant Blinatumomab. Standard: The standard was a mixture of four different proteins at defined concentrations. It was used to construct a standard curve. Intercellular fluid- Blinatumomab: The his-affinity purified blinatumomab monomers (M) and corresponding aggregates (A). X-axis represents the elution time, indicating when different components of the sample are detected. Y-axis represents the signal intensity, which correlates with the amount of a specific compound detected.

For further investigation, polypeptides at the position around ~55 kDa (related to IF-BLIN) were analyzed via peptide mass fingerprinting and the results confirmed the integrity of the IF-BLIN (Fig. 4).

Fig. 4. Peptide mass fingerprinting-based characterization and sequence coverage of recombinant Blinatumomab (extracted from intercellular fluid). The sequence highlighted in gray boxes indicates the covered sequence, and the observed individual peptides are represented as blue lines underlying their respective matching sequence; carbamidomethylated cysteines and oxidized methionines are highlighted in red and orange, respectively.

Attempts to purify BLIN from TSP resulted in insufficient yield and purity (Fig. 5).

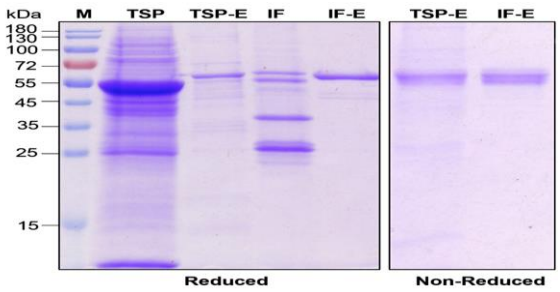

Fig. 5. SDS-PAGE of purified Blinatumomab. A total of 10 µL of each purified Blinatumomab sample was loaded under

reducing conditions. M: protein ladder (4 µL); TSP: Total soluble protein of infiltrated leaf; TSP-E: His purified Blinatumomab from TSP; IF: Intercellular fluid from infiltrated leaf; IF-E: His purified Blinatumomab from IF, Non reduced: TSP-E: His purified Blinatumomab from TSP; IF-E: His purified Blinatumomab from IF.

The figure indicates that purification from the intercellular fluid (IF) yields a higher purity of BLIN compared to purification from total soluble protein (TSP).

These results confirm the successful purification of blinatumomab and provide an accurate representation of the distribution of monomeric and aggregated forms. The analysis of these fractions will be crucial for evaluating the structural integrity and functionality of the purified protein. The degraded products might be related to the endogenous plant proteases, such as those found in *N. benthamiana*, which significantly reduce the yield and bioactivity of recombinant proteins (Benchabane et al., 2008).

The results demonstrate that utilizing the secretory pathway can prevent degradation, thereby enhancing protein stability (liu et al., 2022). Furthermore, using secretory pathway increases the efficiency of His-tag purification from IF samples, highlighting its advantages for improving the recovery of functional recombinant proteins.

Functional activity of plant-derived Blinatumomab

To determine the affinity of plant-derived BLIN from IF to CD19, prior to antigen binding assays, titration experiments were performed. The results detected an IF-BLIN binding affinity of 2.29 nM, whereas FMC63 showed a 7-fold higher affinity with a K_D value of 0.32 nM (Fig. 6).

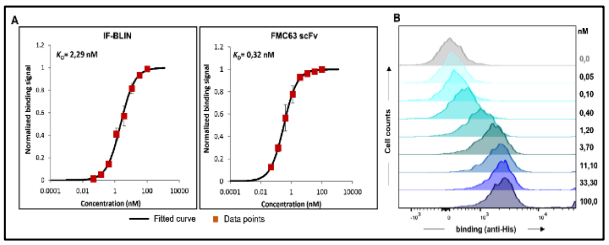

Fig. 6. Titration of Intercellular Fluid- Binatumomab against CD19-positive Nalm6 cells in two-fold serial dilutions. (A) binding curve of Intercellular Fluid-Binatumomab and FMC63 scFv to CD19-positive Nalm6 cells. X-axis shows the concentration of Intercelluar F-BLIN and FMC63 scFv (increasing from low to high). Y-axis represents the extent of binding, expressed as the percentage of cells bound. The red squares represent the proportion of target cells that successfully bound to the analyte at each concentration.

These experimental data are used to calculate K_D. (B) The histograms representing flow cytometry results with a binding pattern of two-fold serial dilutions of IF-BLIN. Flow cytometry histograms display the distribution of binding events at each concentration of IF-BLIN and represents how IF-BLIN binds to the CD19-positive Nalm6 cells at a specific concentration (from the serial dilutions which halved at each step).

To further analyze the binding of IF-BLIN to CD3 and CD19, CD3-expressing Jurkat and CD19 Raji cells were used. Binding to both cell lines was detected, thus confirming that IF-BLIN recognizes both CD3 and CD19 (Fig. 7).

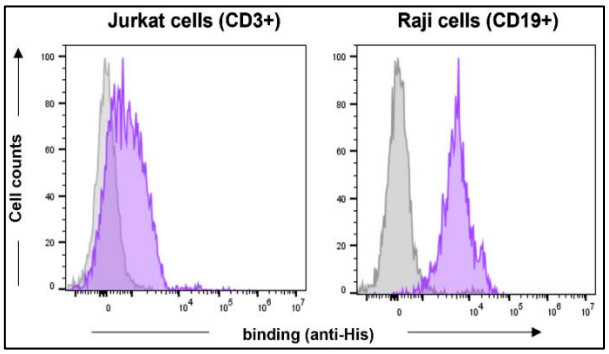

Fig. 7. Binding of Intercellular fluid-Blinatumomab to CD3 and CD19 antigens. Indirect surface staining was performed on Jurkat (CD3-positive and CD19-negative) and Raji (CD19-positive and CD3-negative) cell lines. Purple histograms show the cells stained with Intercellular fluid- BLIN and grey histograms indicate cells without Intercellular fluid - Blinatumomab stained with secondary reagent only. In a histogram plot, the cell count is represented on the Y-axis and the binding signal of the anti-His antibody is on the X-axis.

Plant-based platforms (from whole plant to plant cells) have emerged as an effective platform for producing recombinant proteins, especially for antibodies and their derivatives (Chen, 2022). To produce antibody fragments such as ScFvs, considerable attempts have been made (Galeffi et al., 2005; Gomes et al., 2019; Lai et al., 2014). Fab, ScFvs, and other antibody fragments such as these molecules were generated by exploiting the modular nature of immunoglobulins. Since such molecules are smaller in size compared to fully assembled antibodies, they provide better tissue penetration and usually do not need specific glycosylation for therapeutic efficacy. Hence, it is possible to express them in a system with reduced post-translational modification capabilities. However, some production challenges associated with such molecules are low expression levels, reduced stability, the tendency to form aggregates, and difficult purification (Pirkalkhoran et al., 2023).

In the present study, an attempt was made to produce bispecific T-cell engager BLIN in *N. benthamiana* using potent viral vector-based transient expression. BLIN was successfully expressed as determined by Western Blotting. Upon co-expression of the p19 viral suppressor from tomato bushy stunt virus (Garabagi et al., 2012), BLIN showed a clear increase in expression levels, though a precise quantification was not done here. Nevertheless, compared to full mAbs the expression level of BLIN was low. One of the approaches to increase the antibody fragments' expression level is using ER retention signals such as KDEL or HDEL. Several studies have demonstrated that ER retention with C-terminal HDEL and/or KDEL signals helps improve protein folding. It also

reduces proteolytic degradation related to proteases present in the cytoplasm (Moghadam et al., 2016; Jiang et al., 2019). Also, a recent study revealed that one reason for the low yield of BiTEs is the reduced intracellular mRNA levels encoding the BiTE molecule. The level of transcription could be increased through modifying the primary DNA sequence (Jerabek et al., 2024).

Lack of the Fc domain causes instability and a tendency to form aggregates of antibody fragments such as ScFvs. The Fc domain is known as an essential element to stabilize antibodies (Chen et al., 2013; Zhang et al., 2010). Some studies reported that using *E. coli* known as a high-yield expression platform also faces some challenges like improper folding, insolubility, and formation of inclusion bodies. These challenges limit this platform to producing a fully functional protein (Naddafi et al., 2018; Wang et al., 2019).

Here, we used the alpha-amylase signal peptide for BLIN secretion to the apoplast. Despite carrying signal sequences, not all proteins expressed in plants are secreted to the apoplast (intercellular space). The secretion of proteins depends on protein folding, structure, and size of the molecule. Smaller molecules of 30-50 kDa (A1AT, EPO & Ig5FN1) are secreted well into the apoplast (Castilho et al., 2013; Castilho et al., 2014; Kallolimath et al., 2016). The most complex protein detected in sufficient amounts in the IF was Aflibercept with a theoretical mass of 110 kDa (Keshvari et al., 2024).

Affinity purification of His-tagged proteins from plant TSP extract is challenging; factors affecting yield may include low expression level of the target molecule and non-specific binding of immobilized metal affinity chromatography matrix (Ni-NTA Sepharose) to host cell proteins carrying histidine patches due to its relatively low selectivity (Bornhorst & Falke, 2000). To overcome this challenge, we isolated IF for purification purposes. This fraction carries significantly fewer host cell proteins, resulting in more efficient target protein purification compared to TSP (Fig. 5). The yield of BLIN purified from intercellular fluid (IF-BLIN) was 15 µg/g fresh leaf weight, which is 10 times less compared to the full-length antibodies (Kallolimath et al., 2020). In comparison, CHO cells and *E. coli*-produced BLIN yielded 2 mg/L and 100 mg/L, respectively (Montero-Morales & Steinkellner). However, it should be noted that high production does not always lead to a highly active molecule, as shown for *E. coli*-produced BLIN.

Another approach to improve purification yield would be designing IgG-Fc-based fusion proteins. Attempts were made to design a BiTE with an extended half-life by fusing it to the Fc domain (Goldstein et al., 2020). This approach might improve product recovery by protein-A-based affinity purification. In the case of BiTE or bispecific molecules, the Fc fusion construct can be considered useful to gain Fc-based effector functions often missing in bispecific molecules. However, the increased size of Fc fusion proteins may impact tissue penetration.

In the present study, we used FMC63 ScFv as a positive control to assess the binding efficiency of IF-BLIN to CD19. The assay with FMC63 ScFv has been optimized previously to exclude artifacts due to insufficient equilibration times, ligand depletion, and avidity effects on the measured affinity as reported (Seigner et al., 2023). CD19 domain of BLIN was derived from murine IgG HD37 (Loffler et al., 2000), while the FMC63 ScFv was derived from murine IgG FMC63 (Nicholson et al., 1997). Both molecules bind to the CD19 target antigen with different K_D values. In comparison to FMC63 ScFv, plant-derived IF-BLIN had a 7-fold higher K_D value (2.32 nM). This value is in range with another K_D value of mammalian cell-produced BLIN (1.0 nM) (Dreier et al., 2002). The binding affinity of IF-BLIN to CD3 was 100-fold lower compared to CD19. Therefore, we only conducted staining of CD19-positive cells without titration experiments.

CONCLUSION

We successfully expressed BLIN in plants. It became functionally active as shown by binding to target antigens CD19 and CD3. However, the yield of the purified product was about 10-fold lower compared to fully assembled antibodies expressed similarly. Despite encouraging results, further studies to improve expression levels and purification methods to obtain better yields are envisaged.

FUNDING

Saeideh Dianatkhah has been awarded a scholarship by the Ministry of Sciences, Research and Technology and Shiraz University (Iran) to carry out experiments at BOKU University. Further support: Austrian Science Fund appointed to SK (grant P-37211).

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Saeideh Dianatkhah: Data curation, formal analysis, investigation, and manuscript writing; Chaitra Hiremath: Investigation, formal analysis, and manuscript writing; Stanislav Melnik: Data curation, formal analysis, and manuscript writing; Lin Sun: Data curation and formal analysis; Ali Niazi: Supervision and manuscript editing and improving; Alireza Afsharifar: Supervision and manuscript editing and improving; Amin Ramezani: Conceptualization, supervision, and manuscript writing; Ali Moghadam: Conceptualization, supervision, and manuscript writing; Somanath Kallolimath: Conceptualization, formal analysis, manuscript writing, supervision, and funding acquisition; All authors have read and agreed to the final version of the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL STATEMENT

Saeideh Dianatkhah was awarded a scholarship by the Ministry of Sciences, Research and Technology and Shiraz University (Iran) to carry out experiments at BOKU University. Further support: Austrian Science Fund appointed to SK (P37211 / grant doi: 10.55776/P37211).

DATA AVAILABILITY

Data will be made available on reasonable request from the corresponding author.

ACKNOWLEDGMENTS

MagnICON® vectors were provided by Victor Klimyuk (Icon Genetics GmbH). We thank Prof. Herta Steinkellner for providing valuable discussions. The CytoFLEX S instruments were kindly provided by the EQ-BOKU VIBT GmbH, and the project was supported by the BOKU Core Facility for Biomolecular & Cellular Analysis. Mass spectrometry measurements were performed by Clemens Grünwald-Gruber and Daniel Maresch, Core of Facility Mass Spectrometry, BOKU University, Vienna, Austria.

REFERENCES

Amirzadeh, N., Moghadam, A., Niazi, A., & Afsharifar, A. (2023). Recombinant anti-HIV MAP30, a ribosome inactivating protein: Against plant virus and bacteriophage. *Scientific Reports*, *13*(1), 2091. https://doi.org/10.1038/s41598-023-29365-7.

Asghar, N., Melik, W., Paulsen, K. M., Pedersen, B. N., Bø-Granquist, E. G., Vikse, R., Stuen, S., Andersson, S., Strid, Å., Andreassen, Å. K., & Johansson, M. (2022). Transient expression of flavivirus structural proteins in *Nicotiana benthamiana*. *Vaccines*, *10*(10), 1667. https://doi.org/10.3390/vaccines10101667.

Balieu, J., Jung, J. W., Chan, P., Lomonossoff, G. P., Lerouge, P., & Bardor, M. (2022). Investigation of the N-glycosylation of the SARS-CoV-2 S protein contained in VLPs produced in Nicotiana benthamiana. *Molecules*, *27*(16). https://doi.org/10.3390/molecules27165119

Benchabane, M., Goulet, C., Rivard, D., Faye, L., Gomord, V., & Michaud, D. (2008). Preventing unintended proteolysis in plant protein biofactories. *Plant Biotechnology Journal*, *6*(6), 633–648. https://doi.org/10.1111/j.1467-7652.2008.00344.x

Bornhorst, J. A., & Falke, J. J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology*, *326*, 245-254.

Burt, R., Warcel, D., & Fielding, A. K. (2019). Blinatumomab, a bispecific B-cell and T-cell engaging antibody, in the treatment of B-cell malignancies. *Human Vaccines & Immunotherapeutics*, 15(3), 594-602. https://doi.org/10.1080/21645515.2018.1540828

Castilho, A., Neumann, L., Gattinger, P., Strasser, R., Altmann, F., & Steinkellner, H. (2014). Proteolytic and N-glycan processing of human alpha1-antitrypsin expressed in Nicotiana benthamiana. *Plant Physiology*, *166*(4), 1839–1851. https://doi.org/10.1104/pp.114.250720

Castilho, A., Neumann, L., Gattinger, P., Strasser, R., Vorauer-Uhl, K., Sterovsky, T., Altmann, F., & Steinkellner, H. (2013). Generation of biologically active multi-sialylated recombinant human EPOFc in plants. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0054836

Castilho, A., Strasser, R., Stadlmann, J., Grass, J., Jez, J., Gattinger, P., Kunert, R., Quendler, H., Pabst, M., Leonard, R., Altmann, F., & Steinkellner, H. (2010). In planta protein sialylation through overexpression of the respective mammalian pathway. *Journal of Biological Chemistry*, *285*(21), 15923.15930. https://doi.org/10.1074/jbc.M109.088401

Chen, X., Zaro, J. L., & Shen, W. C. (2013). Fusion protein linkers: Property, design and functionality. *Advanced Drug Delivery Reviews*, *65*(10), 1357-1369. https://doi.org/10.1016/j.addr.2012.09.039

Chen, Q. (2022). Development of plant-made monoclonal antibodies against viral infections. *Current Opinion in Virology*, *52*, 148-160. https://doi.org/10.1016/j.coviro.2021.12.005

Coates, R. J., Young, M. T., & Scofield, S. (2022). Optimising expression and extraction of recombinant proteins in plants. *Frontiers in Plant Science*, *13*, 1074531. https://doi.org/10.3389/fpls.2022.1074531

Dhillon, S. (2023). Elranatamab: First approval. *Drugs*, *83*(17), 1621-1627. https://doi.org/10.1007/s40265-023-01954-w

Dreier, T., Lorenczewski, G., Brandl, C., Hoffmann, P., Syring, U., Hanakam, F., Kufer, P., Riethmuller, G., Bargou, R., & Baeuerle, P. A. (2002). Extremely potent, rapid and costimulation-independent cytotoxic T-cell response against lymphoma cells catalyzed by a single-chain bispecific antibody. *International Journal of Cancer*, *100*(6), 690-697. https://doi.org/10.1002/ijc.10557

Eidenberger, L., Eminger, F., Castilho, A., & Steinkellner, H. (2022). Comparative analysis of plant transient expression vectors for targeted N-glycosylation. *Frontiers in Bioengineering and Biotechnology*, *10*, 1073455. https://doi.org/10.3389/fbioe.2022.1073455

Eidenberger, L., Kogelmann, B., & Steinkellner, H. (2023). Plant-based biopharmaceutical engineering. *Nature Reviews Bioengineering*, *1*(6), 426-439. https://doi.org/10.1038/s44222-023-00044-6

Galeffi, P., Lombardi, A., Donato, M. D., Latini, A., Sperandei, M., Cantale, C., & Giacomini, P. (2005). Expression of single-chain antibodies in transgenic plants. *Vaccine*, *23*(15), 1823-1827. https://doi.org/10.1016/j.vaccine.2004.11.025

Goldstein, R. L., Goyos, A., Li, C. M., Deegen, P., Bogner, P., Sternjak, A., Thomas, O., Klinger, M., Wahl, J., Friedrich, M., & Rader, C. (2020). AMG 701 induces cytotoxicity of multiple myeloma cells and depletes plasma cells in cynomolgus monkeys. *Blood Advances*, *4*(17), 4180-4194. https://doi.org/10.1182/bloodadvances.2020002565

Gomes, M., Alvarez, M. A., Quellis, L. R., Becher, M. L., Castro, J. M. de A., Gameiro, J., Caporrino, M.

C., Moura-da-Silva, A. M., & Santos, M. de O. (2019). Expression of an scFv antibody fragment in *Nicotiana benthamiana* and in vitro assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*. *Toxicon*, *160*, 38–46. https://doi.org/10.1016/j.toxicon.2019.02.011

Hutchings, M., Morschhauser, F., Iacoboni, G., Carlo-Stella, C., Offner, F. C., Sureda, A., Salles, G., Martínez-Lopez, J., Crump, M., Thomas, D. N., Morcos, P. N., Ferlini, C., Bröske, A.-M. E., Belousov, A., Bacac, M., Dimier, N., Carlile, D. J., Lundberg, L., Perez-Callejo, D., Umaña, P., Moore, T., Weisser, M., & Dickinson, M. J. (2021). Glofitamab, a novel, bivalent CD20-targeting T-cell-engaging bispecific antibody, induces durable complete remissions in relapsed or refractory B-cell lymphoma: A phase I trial. *Journal of Clinical Oncology*, *39*(18), 1959–1970. https://doi.org/10.1200/JCO.20.03175

Kallolimath, S., Hackl, T., Gahn, R., Grunwald-Gruber, C., Zich, W., Kogelmann, B., Lux, A., Nimmerjahn, F., & Steinkellner, H. (2020). Expression profiling and glycan engineering of IgG subclass 1-4 in Nicotiana benthamiana. *Frontiers in Bioengineering and Biotechnology*, *8*, 825.

Kallolimath, S., Sun, L., Palt, R., Föderl-Höbenreich, E., Hermle, A., Voss, L., Kleim, M., Nimmerjahn, F., Gach, J. S., Hitchcock, L., Chen, Q., Melnik, S., Eminger, F., Lux, A., & Steinkellner, H. (2024). IgG1 versus IgG3: Influence of antibody-specificity and allotypic variance on virus neutralization efficacy. *Frontiers in Immunology*, *15*, 1490515. https://doi.org/10.3389/fimmu.2024.1490515

Keam, S. J. (2023). Talquetamab: First approval. *Drugs*, *83*(15), 1439-1445. https://doi.org/10.1007/s40265-023-01945-x

Keshvari, T., Melnik, S., Sun, L., Niazi, A., Aram, F., Moghadam, A., Kogelmann, B., Wozniak-Knopp, G., Kallolimath, S., Ramezani, A., & Steinkellner, H. (2024). Efficient expression of functionally active aflibercept with designed N-glycans. *Antibodies*, *13*(2), 29. https://doi.org/10.3390/antib13020029

Lai, H., He, J., Hurtado, J., Stahnke, J., Fuchs, A., Mehlhop, E., Gorlatov, S., Loos, A., Diamond, M. S., & Chen, Q. (2014). Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants. *Plant Biotechnology Journal*, *12*(8), 1098–1107. https://doi.org/10.1111/pbi.12217

Liu, H., & Timko, M. P. (2022). Improving protein quantity and quality—The next level of plant molecular farming. *International Journal of Molecular Sciences*, *23*(3), 1326. https://doi.org/10.3390/ijms23031326.

Loffler, A., Kufer, P., Lutterbuse, R., Zettl, F., Daniel, P. T., Schwenkenbecher, J. M., Riethmuller, G., Dorken, B., & Bargou, R. C. (2000). A recombinant bispecific single-chain antibody, CD19 x CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. *Blood*, *95* (6), 2098-2103.

Maghsoodi, N., Zareinejad, M., Golestan, A., Mahmoudi Maymand, E., & Ramezani, A. (2023). Anti-CD19/CD8 bispecific T cell engager for the potential treatment of B cell malignancies. *Cellular Immunology*, *393–394*, Article 104787. https://doi.org/10.1016/j.cellimm.2023.104787

Moghadam, A., Niazi, A., Afsharifar, A., & Taghavi, S. M. (2016). Expression of a recombinant anti-HIV and anti-tumor protein, MAP30, in nicotiana tobacum hairy roots: A pH-stable and thermophilic antimicrobial protein. *PloS one*, *11*(7), e0159653. https://doi.org/10.1371/journal.pone.0159653

Montero-Morales, L., & Steinkellner, H. (2018). Advanced plant-based glycan engineering. *Frontiers in Bioengineering and Biotechnology*, *6*, 81. https://doi.org/10.3389/fbioe.2018.00081

Moreau, P., Garfall, A. L., van de Donk, N. W. C. J., Nahi, H., San-Miguel, J. F., Oriol, A., Nooka, A. K., Martin, T., Rosinol, L., Chari, A., Karlin, L., Benboubker, L., Mateos, M.-V., Bahlis, N., Popat, R., Besemer, B., Martínez-López, J., Sidana, S., Delforge, M., &Usmani, S. Z. (2022). Teclistamab in relapsed or refractory multiple myeloma. *New England Journal of Medicine*, *387*(6), 495–505. https://doi.org/10.1056/NEJMoa2203478

Naddafi, F., Shirazi, F. H., Talebkhan, Y., Tabarzad, M., Barkhordari, F., Aliabadi Farahani, Z., Bayat, E., Moazzami, R., Mahboudi, F., & Davami, F. (2018). A comparative study of the bispecific monoclonal antibody, blinatumomab expression in CHO cells and E. coli. *Preparative Biochemistry & Biotechnology*, *48*(10), 961–967. https://doi.org/10.1080/10826068.2018.1525562

Nicholson, I. C., Lenton, K. A., Little, D. J., Decorso, T., Lee, F. T., Scott, A. M., Zola, H., & Hohmann, A. W. (1997). Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma. *Molecular Immunology*, *34* (16–17), 1157-1165. https://doi.org/10.1016/s0161-5890(97)00144-2

Phoolcharoen, W., Prehaud, C., van Dolleweerd, C. J., Both, L., da Costa, A., Lafon, M., & Ma, J. K. (2017). Enhanced transport of plant-produced rabies single-chain antibody-RVG peptide fusion protein across an in cellulo blood-brain barrier device. *Plant Biotechnology Journal*, *15*(10), 1331-1339. https://doi.org/10.1111/pbi.12719

Pirkalkhoran, S., Grabowska, W. R., Kashkoli, H. H., Mirhassani, R., Guiliano, D., Dolphin, C., & Khalili, H. (2023). Bioengineering of antibody fragments: Challenges and opportunities. *Bioengineering*, *10*(2), https://doi.org/10.3390/bioengineering10020122

Ravanrouy, F., Niazi, A., Moghadam, A., & Taghavi, S. M. (2021). MAP30 transgenic tobacco lines: from silencing to inducing. *Molecular Biology Reports*, *48*(10), 6719-6728. https://doi.org/10.1007/s11033-021-06662-w

Safonov, A. M., Altunina, A. V., Kolpashnikov, I. S., Solovyeva, D. O., & Oleynikov, V. A. (2025). Application of 3D imaging in biomedical research. *Russian Journal of Bioorganic Chemistry*, *51*(4), 1453-1470. https://doi.org/10.1134/S1068162024607043

Sanford, M. (2015). Blinatumomab: First global approval. *Drugs*, *75*(3), 321-327. https://doi.org/10.1007/s40265-015-0356-3

Seber Kasinger, L. E., Dent, M. W., Mahajan, G., Hamorsky, K. T., & Matoba, N. (2019). A novel anti-HIV-1 bispecific bNAb-lectin fusion protein engineered in a plant-based transient expression system. *Plant Biotechnology Journal*, *17*(8), 1646-1656. https://doi.org/10.1111/pbi.13090

Seigner, J., Zajc, C. U., Dötsch, S., Eigner, C., Laurent, E., Busch, D. H., Lehner, M., Traxlmayr, M. W. (2023). Solving the mystery of the FMC63-CD19 affinity. *Scientific Reports*, *13*(1), 23024. https://doi.org/10.1038/s41598-023-48528-0

Stanciu-Herrera, C., Morgan, C., & Herrera, L. (2008). Anti-CD19 and anti-CD22 monoclonal antibodies increase the effectiveness of chemotherapy in Pre-B acute lymphoblastic leukemia cell lines. *Leukemia Research*, 32(4), 625-632.

Hutchings, M., Morschhauser, F., Iacoboni, G., Carlo-Stella, C., Offner, F. C., Sureda, A., Salles, G., Martínez-Lopez, J., Crump, M., Thomas, D. N., Morcos, P. N., Ferlini, C., Bröske, A.-M. E., Belousov, A., Bacac, M., Dimier, N., Carlile, D. J., Lundberg, L., Perez-Callejo, D., Umaña, P., Moore, T., Weisser, M., & Dickinson, M. J. (2021). Glofitamab, a novel, bivalent CD20-targeting T-cell-engaging bispecific antibody, induces durable complete remissions in relapsed or refractory B-cell lymphoma: A Phase I trial. *Journal of Clinical Oncology*, *39*(18), 1959–1970. https://doi.org/10.1200/JCO.20.03175

Thoreau, F., & Chudasama, V. (2022). Enabling the next steps in cancer immunotherapy: from antibody-based bispecifics to multispecifics, with an evolving role for bioconjugation chemistry. *RSC Chemical Biology*, *3*(2), 140-169.

Wang, Q., Chen, Y., Park, J., Liu, X., Hu, Y., Wang, T., McFarland, K., & Betenbaugh, M. J. (2019). Design and production of bispecific antibodies. *Antibodies (Basel, Switzerland)*, *8*(3), 43.

Wu, J., Fu, J., Zhang, M., & Liu, D. (2015). Blinatumomab: A bispecific T cell engager (BiTE) antibody against CD19/CD3 for refractory acute lymphoid leukemia. *Journal of Hematology & Oncology*, *8*, 104. https://doi.org/10.1186/s13045-015-0195-4

Zaninelli, S., Panna, S., Tettamanti, S., Melita, G., Doni, A., D'Autilia, F., Valgardsdottir, R., Gotti, E., Rambaldi, A., Golay, J., & Introna, M. (2024). Functional activity of cytokine-induced killer cells enhanced by CAR-CD19 modification or by soluble bispecific antibody blinatumomab. *Antibodies (Basel)*, *13*(3), 71. https://doi.org/10.3390/antib13030071.

Zhang, J., Carter, J., Siu, S., W O'Neill, J., H Gates, A., Delaney, J., & Mehlin, C. (2010). Fusion partners as a tool for the expression of difficult proteins in mammalian cells. *Current pharmaceutical biotechnology*, *11*(3), 241-245. https://doi.org/10.2174/138920110791111898