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Vector Design, Cloning, and Expression of a Recombinant Immunotoxin Against Vascular Endothelial Growth Factor Containing the Recombinant Anti-Tumor Protein MAP30 in *Escherichia coli*

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Abstract

Age-related Macular Degeneration (AMD) heavily relies on anti-VEGF therapies, but frequent injections and resistance demand novel approaches. Recombinant immunotoxins present a promising alternative by directly targeting and eliminating pathologically active cells. This study aims to design, clone, and evaluate the expression of a novel anti-VEGF-MAP30 immunotoxin in *Escherichia coli*. The sequence of Ranibizumab (DrugBank: DB01270) was fused to the MAP30 sequence. Following codon optimization, the 1373 bp construct was synthesized and cloned into the pET-28a(+) expression vector. The recombinant plasmid was transformed into five *E. coli* strains (BL21(DE3), DH5α, SHuffle T7, XL1-Blue, and Rosetta-gami). Expression was induced using 1 mM IPTG and evaluated via SDS-PAGE across different time intervals (1 to 4 hours post-induction). Sequencing and gel electrophoresis confirmed the successful cloning of the gene. Among the evaluated hosts, *E. coli* BL21(DE3) exhibited the highest capacity for recombinant protein production, presenting a distinct band at 131 kDa on SDS-PAGE, whereas other strains demonstrated lower expression levels. The successful design and optimal expression of the anti-VEGF-MAP30 immunotoxin in *E. coli* BL21(DE3) provides a foundational step toward developing targeted therapies for ocular angiogenesis and solid tumors. Future purification and *in vitro* assays are warranted to validate its biological efficacy.

Keywords: Cancer, *Escherichia coli*, Immunotoxin, MAP30 toxin, VEGF.

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1. Introduction

Cancer remains a leading cause of mortality worldwide, driving extensive biological studies to discover appropriate and targeted therapies. Tumor growth and metastasis inherently rely on angiogenesis, the formation of new blood vessels (1, 2). This process is activated via an "angiogenic switch" (3, 4), primarily triggered when tumor cells overexpress pro-angiogenic factors, most notably the Vascular Endothelial Growth Factor (VEGF) (5). VEGF-A is recognized as the most potent

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inducer of angiogenesis, and its interaction with the VEGFR2 receptor is pivotal in tumor vascularization (6). Furthermore, uncontrolled angiogenesis driven by VEGF is the primary pathological mechanism in wet age-related macular degeneration (AMD), a progressive disease leading to irreversible central vision loss (7, 8). Consequently, inhibiting the VEGF signaling pathway is a highly valuable strategy for managing both solid tumors and neovascular ocular diseases (9-11). Ranibizumab, a recombinant humanized monoclonal antibody fragment (Fab), effectively targets VEGF-A isoforms, preventing their binding to receptors and thereby reducing angiogenesis and vascular permeability (12).

To enhance the efficacy of targeted therapies, immunotoxins have emerged as highly specific bifunctional molecules. These chimeric structures consist of a targeting moiety, such as a monoclonal antibody, fused to a potent toxin (13). Upon binding to the specific target antigen, the immunotoxin is internalized, and the toxin moiety catalyzes the destruction of the cell, offering superior potency compared to naked antibodies and reduced non-specific toxicity compared to conventional treatments. Toxins can be derived from bacterial or plant sources, with plant-derived ribosome-inactivating proteins (RIPs) being frequently utilized. MAP30 (*Momordica* anti-HIV protein of 30 kDa) is a type 1 RIP isolated from the *Momordica charantia* plant (14, 15). MAP30 induces cell death and exhibits potent anti-tumor activities by irreversibly inhibiting protein synthesis and inactivating DNA. Fusing an anti-VEGF antibody fragment to the MAP30 toxin presents a novel therapeutic approach to specifically eradicate neo-vascular cells in tumors and wet AMD (16).

The successful translation of such complex recombinant proteins relies heavily on selecting an appropriate expression host. *Escherichia coli* is the most prominent prokaryotic system for large-scale recombinant protein production due to its well-characterized genetics, rapid growth kinetics, cost-effectiveness, and high yield (17). However, achieving optimal expression of complex heterologous proteins can be challenging due to factors such as inclusion body formation and variations in disulfide bond formation capabilities among different strains (18).

Therefore, the present study aimed to design and clone a novel synthetic gene encoding a recombinant immunotoxin comprising an anti-VEGF single-chain antibody fused to the MAP30 toxin. Furthermore, this study sought to comparatively evaluate the growth kinetics and protein expression levels of this novel construct across five widely used *E. coli* strains (BL21(DE3), DH5α, SHuffle T7, XL1-Blue, and Rosetta-gami) to identify the optimal host for maximum immunotoxin production.

2. Materials and Methods

2.1. Materials and Bacterial Strains

All chemical reagents, including LB Miller broth, agar, kanamycin, acrylamide, bis-acrylamide, tris base, sodium dodecyl sulfate (SDS), and isopropyl β-D-1-thiogalactopyranoside (IPTG), were purchased from standard commercial suppliers such as Merck, HiMedia, Bio-Rad, and Sigma-Aldrich. Five *E. coli* host strains, BL21(DE3), DH5α, SHuffle T7, XL1-Blue, and Rosetta-gami, were utilized for comparative evaluation (Table 1).

2.2. In Silico Design and Gene Synthesis

The gene sequence encoding the novel recombinant KSH immunotoxin (1373 bp) was designed by fusing an anti-VEGF single-chain variable fragment (scFv, derived from Ranibizumab) and a flexible linker to the plant-derived ribosome-inactivating protein MAP30. The synthetic gene was optimized for E. coli codon usage and cloned into the pET-28a(+) expression vector (6632 bp) containing a kanamycin resistance marker and a T7 promoter (Table 2). Gene synthesis and cloning were performed by Biomatik (Cambridge, Ontario, Canada). The lyophilized plasmid was reconstituted in TE buffer, and its concentration and purity were verified via spectrophotometry (Pico200, Picodrop, UK) and 0.8% agarose gel electrophoresis.

2.3. Bacterial Transformation and Culture Conditions

Competent cells of the five *E. coli*

Table 1. Bacterial strains and plasmid used in this study for the expression of the anti-VEGF-MAP30 immunotoxin.

Strain / Plasmid	Key Characteristics / Genotype	Application in Study
Plasmids		
pET-28a(+)	Contains T7 promoter, KanR (kanamycin resistance), N-terminal His-tag	Expression vector backbone
Bacterial Strains		
E. coli BL21(DE3)	Deficient in lon and ompT proteases; contains T7 RNA polymerase gene	Recombinant protein expression
E. coli DH5 α	Non-pathogenic; ideal for large and complex plasmids	High-efficiency cloning and expression
E. coli SHuffle T7	Cytoplasmic disulfide bond formation (mutated trxB and gor); expresses periplasmic disulfide isomerase	Expression of disulfide-bonded proteins
E. coli XL1-Blue	Contains lac operon; tetracycline resistant	Routine cloning and expression
E. coli Rosetta-gami	Resistant to kanamycin, tetracycline, streptomycin, and chloramphenicol	Enhancing expression of eukaryotic proteins

strains were prepared using the calcium chloride ($CaCl_2$) method (19). Plasmid transformation was carried out via thermal shock at 42 °C for 60-90 seconds. Transformed cells were selected on LB agar plates supplemented with 100 µg/mL kanamycin. For shake flask cultures, a single colony was inoculated into 5 mL of LB medium (primary culture) and incubated overnight at 37 °C and 180 rpm. Subsequently, 1 mL of the primary culture was transferred to secondary shake flasks containing 100 mL of LB medium with kanamycin and grown at 37 °C and 180 rpm.

2.4. Bioreactor Cultivation

Batch cultivations were performed in a 10-L laboratory-scale bioreactor (Raymand Zist Fanavar Alborz, Karaj, Iran) with a working volume of 10 L. Cultivation temperature was maintained at 32±0.5 °C, and aeration was set at 1 vvm. Dissolved oxygen (DO) was maintained above 30% saturation by adjusting the agitation speed (50 to 500 rpm). The pH was controlled at 7.4±0.5 using 1 N NaOH and 1 N HCl. Antifoam (sterilized silicone oil) was added as necessary to control foaming.

2.5. Growth Kinetics and Analytical Procedures

Bacterial growth was monitored hourly by measuring optical density (OD) at 600 nm using a spectrophotometer (Eppendorf BioPhotometer). Dry cell weight (DCW) was determined by centrifuging 5 mL samples,

Table 2. Key structural elements and functional features of the synthesized recombinant pET-28a(+)-KSH Immunotoxin construct.

Genetic Element	Size (bp)	Biological Function / Description
T7 Promoter	16	High specificity for T7 RNA polymerase; directs transcription
lac Operator	25	Binding site for lac repressor; regulates (inhibits) transcription
Target Gene (Immunotoxin)	1373	Encodes the novel anti-VEGF-MAP30 recombinant protein
Kanamycin Resistance KanR	813	Selectable marker to differentiate transformed vs. non-transformed cells
His-Tag	18	Polyhistidine tract for downstream protein detection and purification
Lac I Gene	1080	Encodes the lac repressor protein
T7 Terminator	47	Signals the termination of transcription
*Xba*I / *Xho*I Sites	-	Restriction enzyme cleavage sites flanking the target gene
f1 Origin	-	Origin of replication for single-stranded DNA production

washing the pellet, and drying at 60 °C to constant weight. Specific growth rates (μ) were calculated across distinct exponential growth phases (20). Total protein concentration was quantified using the Bradford assay with Bovine Serum Albumin (BSA) as the standard (21).

2.6. Protein Induction and SDS-PAGE Analysis

When cultures reached an OD600 of approximately 0.7-1.0, recombinant protein expression was induced by adding IPTG to a final concentration of 1 mM. Samples were collected hourly post-induction, and cells were harvested by centrifugation at 5000 × g for 10 minutes at 4 °C. Protein expression patterns were analyzed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a discontinuous system comprising a 5% stacking gel and a 12% resolving gel (22). Gels were stained with Coomassie Brilliant Blue R-250, and band intensities were quantified via densitometry using ImageJ software.

3. Results

3.1. Validation of Gene Synthesis and Cloning

The synthetic gene encoding the novel anti-VEGF-MAP30 immunotoxin was successfully cloned into the pET-28a(+) expression vector (Figure 1). Spectrophotometric analysis of the reconstituted lyophilized plasmid yielded a concentration of 3.5 ng/μL at 260 nm. Sequence integrity and proper in-frame insertion were verified using Chromas software. Furthermore, 0.8% agarose gel electrophoresis confirmed the presence of the recombinant pET28a(+)-KSH Immunotoxin construct at the expected total size of 6632 bp,

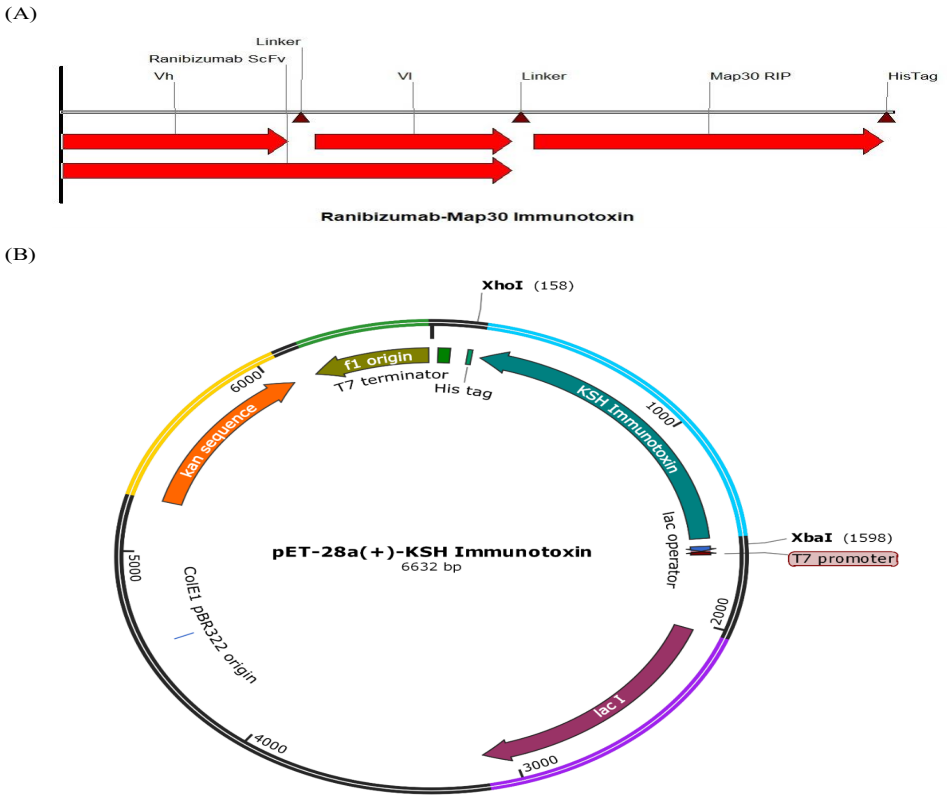


Figure 1. *In silico* design and structural features of the novel anti-VEGF-MAP30 immunotoxin. (A) Schematic representation of the synthetic immunotoxin construct demonstrating the variable heavy (VH) and light (VL) chains of the anti-VEGF scFv (Ranibizumab), connected via flexible linkers to the MAP30 ribosome-inactivating protein and a C-terminal His-tag. (B) The circular map of the 6632 bp recombinant pET-28a(+) expression vector illustrating the insertion of the 1373 bp target gene downstream of the T7 promoter and the kanamycin resistance KanR marker.

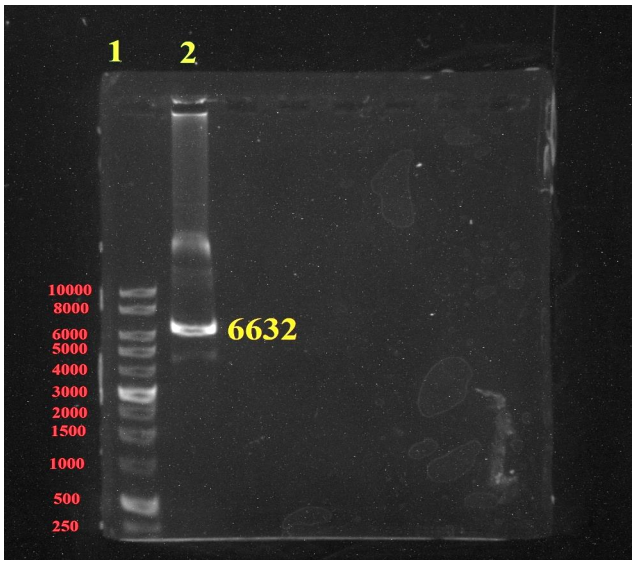


Figure 2. Agarose gel electrophoresis (0.8%) confirming the structural integrity of the recombinant plasmid. Lane 1: 10 kb DNA molecular weight marker; Lane 2: The extracted pET28a(+)-KSH Immunotoxin plasmid from transformed *E. coli* BL21(DE3) exhibiting the expected total size of 6632 bp.

encompassing the 1373 bp target gene (Figure 2).

3.2. Evaluation of Bacterial Transformation

Successful transformation of the recombinant plasmid into the five *E. coli* host strains (BL21(DE3), DH5α, SHuffle T7, XL1-Blue, and Rosetta-gami) was confirmed via selective plating. Transformed colonies exhibited robust growth on LB agar plates supplemented with 100 µg/mL kanamycin, whereas untransformed control cells showed no growth, simultaneously verifying the absence of cross-contamination.

3.3. Growth Kinetics of Recombinant Strains

Growth kinetics were monitored via turbidimetry at 600 nm. Prior to transformation, the untransformed SHuffle strain exhibited the highest baseline growth rate. However, following transformation and induction with 1 mM IPTG, the growth profiles altered significantly. The BL21(DE3) and DH5α strains demonstrated enhanced growth capacities post-transformation. Conversely, the SHuffle and Rosetta-gami strains exhibited decreased growth rates, while the XL1-Blue strain showed no significant alteration. Calculations of the specific growth rate (µ) for the BL21(DE3) strain indicated that maximum growth velocity occurred between 3 and 4 hours post-induction (Table 3). Batch cultivation of the BL21(DE3) strain in a 10-L bioreactor corroborated these findings, demonstrat-

Table 3. Specific growth rate (µ) of *E. coli* BL21(DE3) prior to and following transformation during batch cultivation.

Time Interval (Δt)	Non-Transformed Strain µ (ΔOD/Δt)	Transformed Strain µ (ΔOD/Δt)
T1 – T0	0.060	0.023
T2 – T1	0.105	0.177
T3 – T2	0.191	0.971
T4 – T3	0.638	1.155
T5 – T4	0.508	0.674
T6 – T5	0.110	0.400
T7 – T6	0.161	0.330
T8 – T7	0.173	0.116

Note: The maximum specific growth rate for the transformed strain was observed between T3 and T4, which corresponds to the peak phase of recombinant protein production as confirmed by SDS-PAGE analysis.

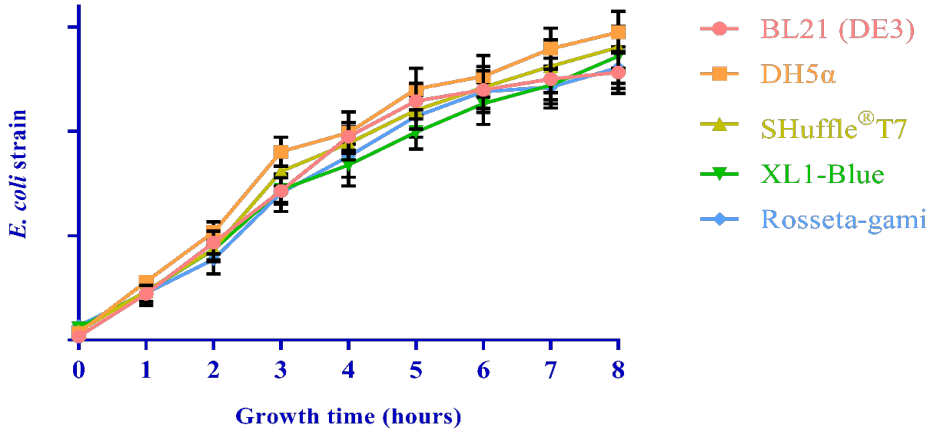


Figure 3. Comparative growth kinetics of the five transformed *E. coli* strains (BL21(DE3), DH5 α, SHuffle T7, XL1-Blue, and Rosetta-gami). The cell density (OD600) was monitored over an 8-hour cultivation period in shake flasks containing LB medium supplemented with kanamycin (100 µg mL-1) following induction with 1 mM IPTG. Data are presented as the mean of three independent experiments ($p < 0.05$).

ing optimal growth kinetics and maximum capacity for recombinant protein production under controlled parameters (Figure 3).

3.4. Expression Analysis of the Recombinant Immunotoxin

Protein expression was evaluated using SDS-PAGE following a 4-hour IPTG induction period. Electrophoretic analysis of the cell lysates across all five recombinant strains revealed a distinct protein band at the predicted molecular weight of 131 kDa, corresponding to the target anti-VEGF-MAP30 immunotoxin. Densitometric analysis utilizing ImageJ software confirmed that the BL21(DE3) strain yielded the highest expression level of the recombinant protein compared to the other evaluated hosts. Time-course analysis of the BL21(DE3) lysates further indicated that the maximum accumulation of the 131 kDa target protein was achieved 3 to 4 hours post-induction (Figure 4).

4. Discussion

Targeted therapies remain at the forefront of managing angiogenesis-dependent pathologies, primarily solid tumors and AMD. The "angiogenic switch," driven largely by the overexpression of VEGF, is critical for tumor progression, metastasis, neovascularization, and immune suppression. While monoclonal antibodies, such as those targeting VEGF or Epidermal Growth Factor Receptors (e.g., Her1/EGFR and HER2), have revolutionized targeted therapy, their clinical efficacy is frequently limited by dose-limiting toxicities, multidrug resistance, and poor tumor penetration. Linking highly specific targeting moieties, such as scFvs or compact single-domain antibodies (sdAbs), to potent cytotoxic payloads creates bifunctional immunotoxins that can overcome drug resistance and minimize non-specific toxicity through catalytic cellular destruction.

Historically, the clinical development of immunotoxins has faced challenges regarding off-target toxicity and dose-limiting immune responses, necessitating the design of highly specific conjugates with wider therapeutic indices. Targeting the VEGF is a well-validated anti-tumor strategy, as VEGF overexpression directly induces pro-angiogenic and immunosuppressive cascades crucial for tumor progression. Previous investigations utilizing anti-VEGFR2 fused with truncated *Pseudomonas* exotoxin A have demonstrated significant synergistic cytotoxicity and increased apoptotic indices in tumor cell lines. This study successfully engineered a novel immunotoxin by conjugating an anti-VEGF targeting moiety to MAP30, a selectively toxic type 1 RIP extracted from the plant *Momordica charantia*, aiming to maintain robust anti-tumor efficacy while limiting toxicity to

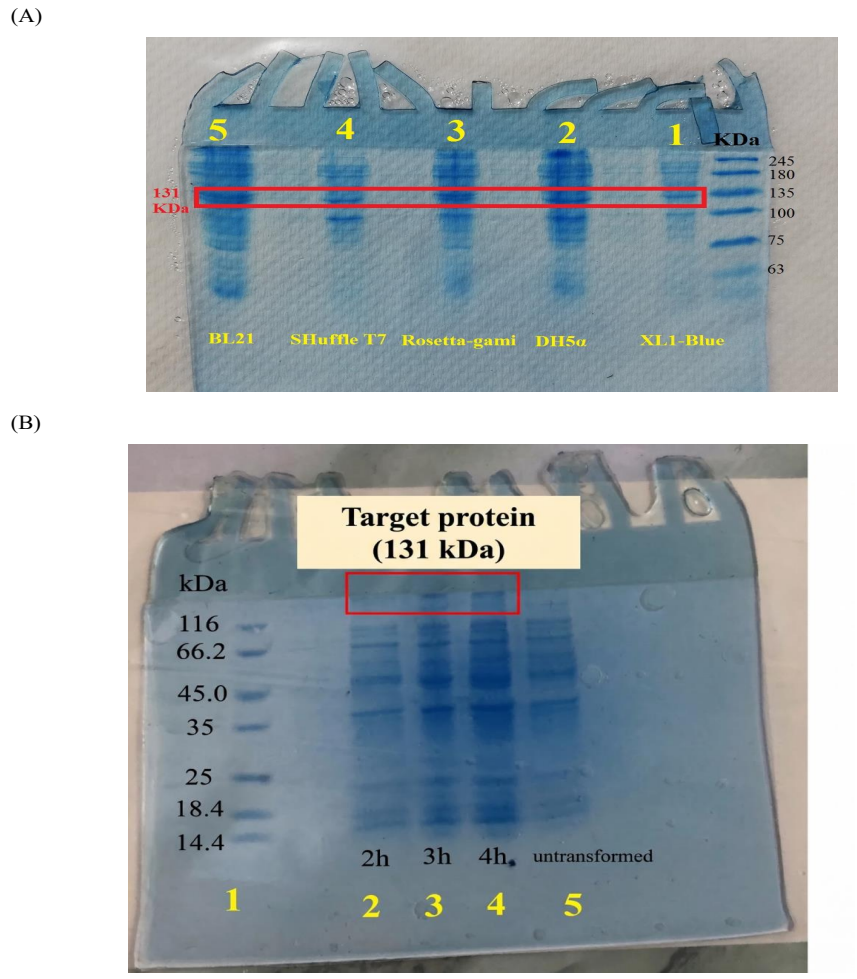


Figure 4. SDS-PAGE (12% resolving gel) analysis of the recombinant anti-VEGF-MAP30 immunotoxin expression. (A) Comparison of protein expression profiles across the five transformed *E. coli* strains 4 hours post-induction. The distinct band at 131 kDa (red box) corresponds to the target immunotoxin, with BL21(DE3) exhibiting the highest expression level. (B) Time-course expression analysis of the BL21(DE3) strain. Lane 1: Protein molecular weight marker; Lanes 2-4: Cell lysates at 2, 3, and 4 hours post-induction with 1 mM IPTG, demonstrating maximum protein accumulation at 3-4 hours; Lane 5: Untransformed BL21 negative control.

normal cells. MAP30 exerts robust anti-tumor and apoptotic effects by irreversibly halting protein synthesis and inactivating DNA. The localized delivery of MAP30 via the anti-VEGF fragment presents a promising strategy to selectively eradicate neo-vascular cells and spare normal tissues.

In previous developments, clinically validated bacterial toxins have been widely utilized to construct these chimeric molecules. For example, the FDA-approved Denileukin diftitox (DAB389IL-2) utilizes a truncated Diphtheria Toxin (DT) to selectively induce apoptosis in T-cell lymphomas via ADP-ribosylation and nuclease activity, as described by Zarkar *et al.* (23), a mechanism also exploited by Ghodrati Siahmazgi et al. using DT389 fused to GCSF for leukemia targeting (24). For solid tumors, optimized *Pseudomonas* Exotoxin A (PE) variants (e.g., PE35KDEL, PE24B, PE24X7) utilized by Shariaty Vaziri et al. (25), Park *et al.* (26), and Xing et al. (27) have demonstrated exceptional potency while significantly reducing immunogenicity and off-target toxicity. Furthermore, bacterial superantigens, such as the Staphylococcal enterotoxin C2 (SEC2) explored by Ming-Kai and Cheng-Gang (28), offer a distinct mechanism by actively stimulating immune cell proliferation alongside direct tumor targeting. Despite their potency, bacterial toxins can provoke severe anti-drug antibody (ADA) re-

sponses. To reduce immunogenicity, researchers have explored human-origin apoptosis-inducing proteins like TRAIL, successfully utilized by Wang et al. (29), engineered mammalian RNase A to evade endogenous inhibitors as shown by Forouharmehr *et al*. (30), or snake venom-derived crotamine for its unique endosomal disruption properties, highlighted by Jang *et al*. (31).

The efficacy of these chimeric molecules heavily depends on their precise structural design and conjugation techniques. In silico structural validation is crucial to ensure that chosen peptide linkers prevent steric hindrance and preserve the binding affinity of the functional domains. Wang *et al*. utilized predictive software to screen linkers based on flexibility and solvent accessibility, determining that a longer (GGGGS)4 linker optimally maintained the functional independence of the scFv and TRAIL domains (29). Similarly, optimized flexible motifs like SerGly4SerMet, variants of the (GGGGS)n sequence, or furin-cleavable sequences are frequently employed to facilitate correct folding and cytosolic toxin release. Advanced linking strategies, such as the FcBP-mediated photoconjugation utilized by Kim *et al.* (32), further improve site-specific conjugation and overall production yields. This underscores the critical importance of the in silico design, linker selection, and structural validation implemented in our novel anti-VEGF-MAP30 construct.

A major translational bottleneck for immunotoxins is identifying an optimal expression host capable of yielding soluble, active proteins while circumventing insoluble inclusion bodies. While functional immunotoxins can be recovered from *E. coli* inclusion bodies via extensive denaturing and refolding protocols, identifying strategies that naturally maximize soluble yield is preferable. To bypass complex *in vitro* refolding, researchers frequently employ solubility-enhancing fusion tags. Park *et al*. utilized a Maltose Binding Protein (MBP) chaperone (26), Ming-Kai and Cheng-Gang (28) employed a Thioredoxin (TRX) tag, and Wang *et al.* (29) utilized a Small Ubiquitin-related Modifier (SUMO) tag to facilitate proper folding in *E. coli*. Moreover, Cao *et al*. (33) demonstrated that coupling an MBP tag with a self-cleaving split intein (Npu DnaE) eliminates the high costs associated with enzymatic tag removal, significantly streamlining downstream purification. While *E. coli* BL21(DE3) remains the universal foundational host, its reductive cytoplasm impedes the formation of critical disulfide bonds. Addressing this, Zarkar *et al.* (23) demonstrated that the *E. coli* Rosetta-gami (DE3) strain is highly effective for producing fully soluble, disulfide-bonded constructs, and Mani et al. optimized cultivation variables (temperature, inducer concentration) using Response Surface Methodology (RSM) to successfully enhance soluble expression (34). By systematically comparing five *E. coli* variants, our study established that the BL21(DE3) strain optimally supports the production of the target immunotoxin, aligning consistently with the foundational hosts utilized across these diverse studies.

Finally, the clinical application of systemically administered immunotoxins can be hindered by off-target toxicity in tissues expressing basal antigen levels, as well as the impracticality of continuous exogenous inducer administration. Pioneering approaches now utilize live tumor-targeting bacteria for localized secretion directly within the hypoxic tumor microenvironment (TME). While Li *et al.* employed *E. coli* Nissle 1917 for localized PD-L1-PE38 delivery using exogenous L-arabinose (35), Hong *et al*. engineered *E. coli* with an auto-inducing csrB promoter that senses TME acetate levels, enabling continuous, tumor-specific expression of a TGFa-PE38 immunotoxin without external chemical triggers (36). The successful scale-up of our transformed BL21(DE3) strain in a 10-L benchtop bioreactor confirms its exceptional suitability for large-scale manufacturing. Future *in vivo* strategies could similarly integrate these engineered probiotics with advanced structural designs to maximize the therapeutic index, localized efficacy, and clinical feasibility of our proposed anti-VEGF-MAP30 immunotoxin.

5. Conclusion

The successful in silico design, cloning, and heterologous expression of the novel

anti-VEGF-MAP30 immunotoxin represents a significant step forward in developing targeted therapies for angiogenesis-dependent pathologies, including solid tumors and wet AMD. By utilizing the plant-derived MAP30 ribosome-inactivating protein, this chimeric construct is strategically designed to maintain potent catalytic cytotoxicity against neovascular cells while potentially mitigating the severe immunogenicity and off-target toxicities typically associated with traditional bacterial toxins.

Among the evaluated expression hosts, *E. coli* BL21(DE3) demonstrated superior bioprocessing metrics, exhibiting optimal growth kinetics and yielding the highest accumulation of the 131 kDa target protein. The successful scale-up of this strain in a 10-L benchtop bioreactor confirms the robustness and scalability of the production process. Future investigations must focus on the rigorous downstream purification of the recombinant protein, followed by comprehensive in vitro cell viability assays and in vivo models to definitively validate its specific binding affinity, apoptotic mechanisms, and overall therapeutic efficacy.

Authors Contributions

KSH: Investigation, Methodology, Formal analysis, Data curation, Writing - original draft. AR: Validation, Methodology, Visualization, Writing - review & editing. YG: Conceptualization, Resources, Supervision, Validation, Writing - review & editing. MHM: Conceptualization, Resources, Project administration, Funding acquisition, Supervision, Writing - review & editing.

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Data availability

Data supporting the findings of this study can be obtained from the corresponding author upon reasonable request.

Ethical approval

The Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC. 1398.070) approved all procedures and protocols.

Declaration of generative AI in scientific writing

During the preparation of this work, the authors used Google Gemini and Grammarly solely to improve the English language, grammar, readability, and formatting of the manuscript and the response to reviewers. After using these tools, the authors closely reviewed and edited the content as needed and take full responsibility for the integrity, accuracy, and final content of the publication.

Conflict of Interest

The authors declare that they have no conflict of interest.

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