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Original Research

Construction and recombinant expression of *Pseudomonas aeruginosa* truncated exotoxin A in *Escherichia coli*

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Abstract: *Pseudomonas aeruginosa* exotoxin A (PE) is a bacterial toxin composed of three domains namely: cell binding, translocation and enzymatic domain. The cytotoxic activity of PE is attributed to the enzymatic domain, which inhibits protein synthesis through ADP-ribosylation of EF-2. PE can be genetically modified to fight cancer. In this regard, a truncated and modified form of PE was produced that could be used for more potent immunotoxins. This modified form termed PE38KDEL was completely devoid of cell binding domain and parts of translocation domain II and Ib which are reported to be inessential for cytotoxicity of the toxin. The resultant expressed protein consisted of the essential translocation domain II and catalytic subunit (domain Ib, III). The deletions in the exotoxin A gene for truncated protein production were made via overlapping PCR extension. The amplicon was cloned in pTZ57r-T vector for DNA works and sub cloned in pET22b expression vector. It is demonstrated here that PE38KDEL can be expressed in huge quantities in *Escherichia coli* by using the recombinant vector PE38KDEL/pET under control of T7 promoter and *E. coli* host strain BL21 (DE3) CodonPlus. The protein expression was optimized at 0.5 mM IPTG concentration for induction as soon as the OD_{600} nm reached 0.6 with 6 hours of post induction culturing at 37°C. The recombinant protein was expressed both as soluble and inclusion body forms however the expression of the soluble form was more pronounced.

Key words: *Pseudomonas aeruginosa* exotoxin A; Cytotoxic, immunotoxin; PE38KDEL; Expression.

Introduction

Cancer accounts for most death rates next to cardiovascular diseases around the globe, with just about 14 million new reports of cancer patients and 8.2 million deaths tied up to it in 2012 (1). The figures for expected cases in future are estimated to accelerate by 70% within the next score (2). Although new drugs to fight cancer have been and are being developed, novel therapeutic strategies are required owing to the higher frequency of drug resistance and toxic side effects resulting from the known treatments (3). The most challenging front in treating cancer for ages is the selective destruction of cancer cells. A promising anticancer agent must be programmed to target cancer cells only, identify them through their highly expressed molecular cell-surface proteins and destroy them efficiently sparing the normal body cells (4). The field of immunotoxins offer such potent selectively targeting cytotoxic agents which are internalized and successively cause cell death (5). An immunotoxin entails an antibody joined to a toxin and is intended to explicitly exterminate tumor cells (6). Protein toxins of bacterial or plant origin are noteworthy in this instance for the severe morbidity and mortality rates they caused as 'single agents'. They may be approached to play toxin moiety role in immunotoxins for sharing a common biochemical mechanism of inhibition of protein synthesis leading to programmed cell death (7, 8). Initially built immunotoxins utilized whole protein toxins but far along they are now based on protein toxins devoid of native binding domains (9).

Exotoxin A of *Psuedomonas aeruginosa* (PE), produced as a virulent factor by many strains, is an extremely toxic protein of 66 kDa having a median lethal dose (LD_{50}) of 200 ng in mice (10). It has been extensively reported that PE can be genetically modified to fighht cancer (11). X ray crystallography of this protein confirmed three structural domains namely: the receptor binding domain I (a.a residues 1–252 and 365–404), the translocation domain II (a.a residues 253–364), and the enzymatic domain III (a.a residues 405–613) (12, 13). The cytotoxic action of the PE is attributed to the enzymatic domain (14). Concerning killing efficiency of the toxin, it was reported that as little as 1000 molecules of PE per cell are sufficient enough to induce cell death *in vitro* and around 400-750 molecules per cell can result in tumor regression *in vivo* (15). Modern improvements for the advancement of recombinant immunotoxins have centered on creating smaller versions of the original *Pseudomonas* Exotoxin A named as PE40/38 that are less immunogenic than their parent protein (16-18). By the elimination of an enormous region from domain II of PE along with completely removed domain I, a truncated molecule have been synthesized that still displayed the cytotoxicity with added benefits of one significant and numerous insignificant immunogenic epitopes deleted (19-23). The protein expressed in this investigation was designed bearing in mind the factors crucial to cy-

Table 1. Sequence of Oligonucleotide Primers.

Primers	Nucleotide Sequence	Length
PE-1	5'- AAA**CCATGG**AGGGCGGCAGCCTGGCCGC-3'	32mer
PE-2	5'-TCGCAACACTAGAAGTACAGCGGCCAGACGGGCCTGCTCCG-3'	41mer
PE-3	5'-GCTGTACTTCTAGTGTTGCGACTGACCTGCCCGGTCGCCGCC-3'	42mer
PE-4	5-**GGATCC**TTACAGTTCGTCTTTGATGCTGGACGGGTCGAGGTCG-3'	43mer

*BamHI=CCATGG *NcoI=GGATCC *Bases underlined and colored grey are the complementary regions.

totoxicity. The aim of the study was to create a truncated and modified toxin protein that could be used for more potent immunotoxins. The extensive modifications in the toxin would redirect its potent cytotoxicity from disease to a therapeutic function. The PE version produced encompassed 277-364 amino acids from domain II region and 395-613 amino acids of domain Ib and III. This PE protein was devoid of complete domain Ia and parts of domain II (253-276) and domain Ib (365-394). Furthermore C-terminus residues (REDLK) were also replaced by ER retention motif 'KDEL'. It has been reported that the last few amino acids residues of PE (REDLK) are very important for cytotoxic activity though not linked to its ADP ribosylation activity (24). Studies have indicated that the change in the amino acid sequence at the carboxyl terminus of PE from REDLK to KDEL, an endoplasmic reticulum retention sequence (25), could keep the molecule fully active (24). This recommended that a mutual factor may be required in cells intoxication by PE and the retention of proteins in the lumen of the endoplasmic reticulum.

Materials and Methods

Culturing of *Pseudomonas aeruginosa* and host microorganisms

Pseudomonas aeruginosa, the source organism for Exotoxin A (ETA/PE) gene, was very kindly provided by the Chugtais Laboratories, Lahore. All the bacteria used in the study were grown on LB agar medium at 37 ºC and stored at 4 ºC with cultures refreshed weekly. They were also preserved as glycerol stock at –20 ºC. The recombinant bacterial strains were taken from School of Biological Science, University of the Punjab, Lhr.

Amplification of PE38KDEL sequence using overlap extension PCR

The genomic DNA was obtained through phenol/chloroform extraction using a modified DNA isolation protocol (26). In this study, *Pseudomonas aeruginosa* Exotoxin A (PE/ETA) gene was truncated by the deletion of complete cell binding domain Ia (1-252 a.a), part of translocation domain II (253-276 a.a) and domain Ib (365-389) coding nucleotide sequences. The resultant gene encoded for reduced translocation domain and essential catalytic domain and termed as PE38KDEL

Two external primers PE-1 and PE-4 and two internal primers PE-2 and PE-3 were designed. Table 1 enlists the sequence of oligonucleotides designed for the PCR amplification. The external forward (5') primer (PE-1) contained an *NcoI* site followed by ATG and the N-terminal sequence of PE gene. The external reverse (3') primer (PE-4) was designed to contain complementary sequence of the C-terminus of PE38KDEL followed by KDEL motif, stop codon and a *Bam HI* site. The inner primers (PE-2, PE-3) had complementary regions. PE gene sequence coding for a.a. residues 277–364 was amplified using primers PE-1 and PE-2 whereas the sequence encoding a.a. residues 395-613KDEL was amplified with the primers PE-3 and PE-4. Both the fragments were then fused together using the overlapping PCR method and outer primers PE-1, PE-4. The final PCR product was the truncated PE38KDEL sequence. The thermo cycling conditions used for each PCR reaction is shown in Table 2. Thermo resistant Taq polymerase (Thermo Fisher) was employed to achieve the amplification. All the PCR products were electrophoresed on 1 % agarose gel, visualized under UV light after staining with ethidium bromide and purified using Gene JET™ Gel extraction kit (Fermentas).

Cloning of PE38KDEL amplified fragment

pTZ57R/T vector (Fermentas) was used for cloning of PCR product (PE38KDEL) with 3´-dA overhangs to form recombinant PE38KDEL/pTZ vector. Ligation mixture was transformed into *Escherichia coli* strain DH5α (27). Blue-white screening, performed manually, was used to identify the recombinant bacteria. The recombinant colonies were further screened by the restriction digestion analysis of miniprepared plasmid DNA with *Eco RI* and *Hind III*.

Table 2. Thermo cycling conditions for amplification of PE38KDEL gene sequence.

Phases	First PCR[1]	Second PCR[2]	Third PCR[3]
Initial denaturation	96°C for 1 min	94°C for 5 min	94°C for 5 min
Denaturation	96°C for 3 min	94°C for 1 min	94°C for 1 min
Annealing	50°C for 30 sec	50°C for 45 sec	55°C for 45 sec
Extension	72°C for 1 min	72°C for 1 min	72°C for 1 min
Final Extension	72°C for 7 min	72°C for 7 min	72°C for 7 min
Hold	4°C	4°C	4°C

[1]First PCR: conditions for amplification of domain II (a.a residues 277-364) coding region of PE gene. [2]Second PCR: conditions for amplification of domain III (a.a. residues 395-613KDEL) coding region of PE gene. [3]Third PCR: conditions for overlap extension PCR.

Sequence analysis

Isolated recombinant PE38KDEL/pTZ plasmid from positive transformants after restriction digestion analysis was subjected to DNA sequencing. Sequencing was done in forward direction using M13 forward primer on ABI PRISM 310 automated capillary genetic analyzer. The electropherogram of sequenced PE38KDEL was initially scrutinized and assessed with Chromas Lite (version 2.1). The acquired nucleotide sequence and the inferred protein sequence of PE38KDEL were subjected to NCBI 'BLASTn' and 'BLASTp' respectively for finding out the homology scores of sequences appearing in BLAST hits.

Cloning of PE38KDEL in expression vector

The sequence confirmed recombinant plasmid PE38KDEL/pTZ was digested with suitable restriction enzymes i.e. *Nco I* (restriction site present at the 5' end of the insert DNA) and *Bam HI* (restriction site present at the 3' end of the insert DNA) for the creation of product with sticky ends. The expression clone 'PE38KDEL/pET' was created by ligating the restricted PE38KDEL fragment with overhangs into expression vector pET22b (also digested with *Nco I* and *Bam HI*). The ligation reaction (PE38KDEL/pET) was transformed into competent cells of *E. coli* DH5α prepared using calcium chloride treatment for further downstream analysis. The transformed mixture was plated on to LB agar plates supplemented with ampicillin (10 µg/mL). Screening for the positive colonies was performed by restriction enzyme digestion with *NcoI* and *BamHI* of miniprepared plasmid DNA (PE38KDEL/pET22b).

Expression of recombinant protein PE38KDEL

The positive clones were transformed into *E. coli* BL21 DE3 and restriction confirmed. The expression was optimized through small scale testing by analyzing the effects of various growth parameters such as; type of inducer (IPTG or Lactose), concentration of inducer and induction time. One or two of colonies of the *E. coli* BL21 DE3 with positive clones and empty expression vector were grown in 10 mL LB cultures containing the appropriate antibiotics at 37 ºC overnight. After 24 hours, 200 µL of these cultures served as inoculum for another 10 mL cultures which were allowed to grow at 37 ºC at 150-170 rpm until cells reached mid log phase (OD_{600} 0.6-0.7). At this point an aliquot (1 mL) from the culture with positive clones was stored on ice as a non-induced control. The remainder of the cultures were induced with various concentrations of IPTG (0.2 mM, 0.5 mM and 1.0 mM) or lactose (2.0 mM, 5.0 mM, 10 .0 mM, 15.0 mM and 20.0 mM) for different time periods (0-6 and 18 hours). While only one variable varied at a time with the others kept constant, 1 mL post induction samples were collected at various time points during the incubation period (e.g., 2, 4, 6 and 18 hours). The cells in the tubes were harvested by centrifugation (Eppendorf, Centrifuge 5430R) at 12000 rpm for 1 minute at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µL of 50 mM Tris-Cl buffer. After the addition of 1X SDS gel loading buffer to the resuspended pellet, the samples were heated to 100 ºC for 3 minutes. The tubes were again centrifuged at 12000 rpm for 1 minute in a microfuge and the cell lysate was stored on ice until ready to load on a gel.

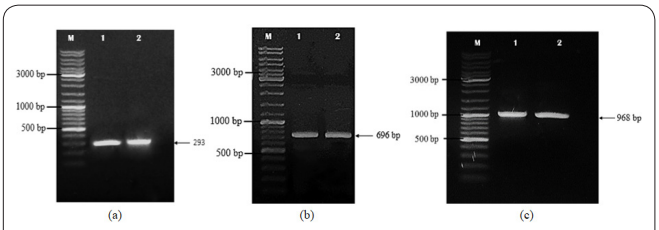

Figure 1. 1% agarose gel of the products of two step overlapping PCR (a) 293 bp gene fragment encoding for translocation domain II (277-364 aa) of PE, (b) 696 bp gene fragment encoding for catalytic subunit of PE (395-613 aa) amplified by PCR. (c) Truncated PE38KDEL gene consisting of reduced translocation domain II (277-364) and catalytic domain III (395-613) amplified using overlap extension PCR. Lane M: Gene Ruler DNA ladder mix (Thermo Scientific #SM0331).

Results

Amplification of PE38KDEL gene sequence by PCR-driven overlap extension

PE38KDEL is a truncated form of exotoxin A of *Pseudomonas aeruginosa* (PE). It was created via extension of overlapping gene segments by PCR. Two suitable primer pairs were designed accordingly. The internal primers i.e. PE-2 and PE-3 had complementary region and acted as a bridge between the two parts that needed to be assembled together. The initial PCRs generated two overlapping gene segments of 293 bp and 696 bp which served as template DNA for a third PCR. Overlapping strands of these intermediate PCR products hybridized at the complementary region in the subsequent PCR and were extended to create a final product of 968 bp amplified by flanking primers i.e. PE-1 and PE-4 (Figure 1).

Cloning and sequencing of PE38KDEL

The minipreparations of the cloned plasmid (PE38KDEL/pTZ) isolated from *E. coli* DH5α were analyzed on 1% agarose gel electrophoresis and subsequently digested with *Eco RI* and *Hind III*. Expected bands for the product of restriction were 2811 bp for pTZ57R/T and 1043 bp for PE38KDEL (Figure 2). After the successful cloning of PCR product into pTZ57R/T vector, the identity of the product as truncated PE38KDEL frag-

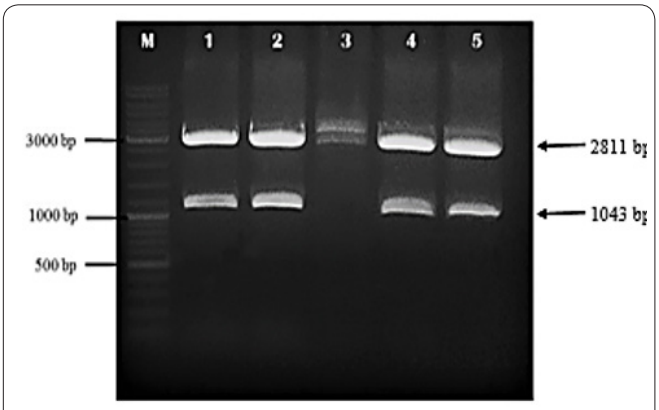

Figure 2. 1% agarose gel of the digested plasmid PE38KDEL/pTZ. Colony screening was carried out by digestion analysis with *Eco RI* and *Hind III*. Lane M represents Gene Ruler DNA ladder mix (Thermo Scientific #SM0331).

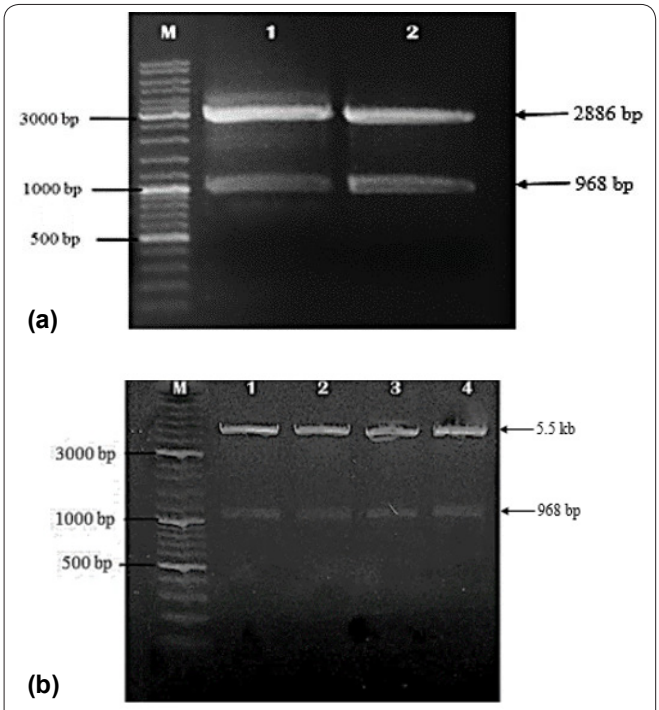

Figure 3. 1% agarose gel of the product of restriction digestion of recombinant PE38KDEL/ pET clones. (a) Digestion was carried out with *Nco I* and *Bam HI*. (b) Screening was carried out by digestion with *Nco I* and *Bam HI.*

ment lacking complete domain I and portions of domain II was confirmed by DNA sequencing.

Cloning of PE38KDEL gene in pET22b expression vector

pET-22b(+) protein expression vector system was utilized to examine the expression of PE38KDEL in *E. coli*. The gene was engineered with *NcoI* and *BamHI* to meet the requirements for sub cloning into expression vector system. It was excised using *NcoI* and *BamHI* from PE38KDEL/pTZ vector. Figure 3a shows results of such restriction digestion. Ligation was an important step for the insertion of digested PE38KDEL into the digested pET22b vector. The ligated vector termed PE38KDEL/pET was transformed into *E. coli* DH5α cells and the positive colonies were once again screened through restriction analysis. The isolated plasmids PE38KDEL/pET from the recombinant colonies obtained on selective LB agar medium were digested with *NcoI* and *BamHI* to ascertain the presence of gene of interest in the pET22b vector (Figure 3b).

Expression of PE38KDEL protein in *E. coli*

E. coli BL21 (DE3) was transformed with PE38KDEL/pET, purified from *E. coli* DH5α, and level of expression was investigated. Once the culture was grown to 0.6 OD, it was induced with IPTG at optimized concentration of 0.5 mM and further allowed to grow for 6 hours post induction at 37 ºC. Cells were then harvested and centrifuged to collect the pellet which was resuspended in 50 mM Tris-Cl (pH 8.0). All the prepared control and induced cultures were analyzed on 12% SDS-PAGE (Figure 4). Figure 4 displays a notable difference in the profile of protein between the lane of bacteria with pET-22b(+) and the one with induced culture of bacteria containing pET22b/PE38KDEL. The presence of the expressed protein with the electrophoretic

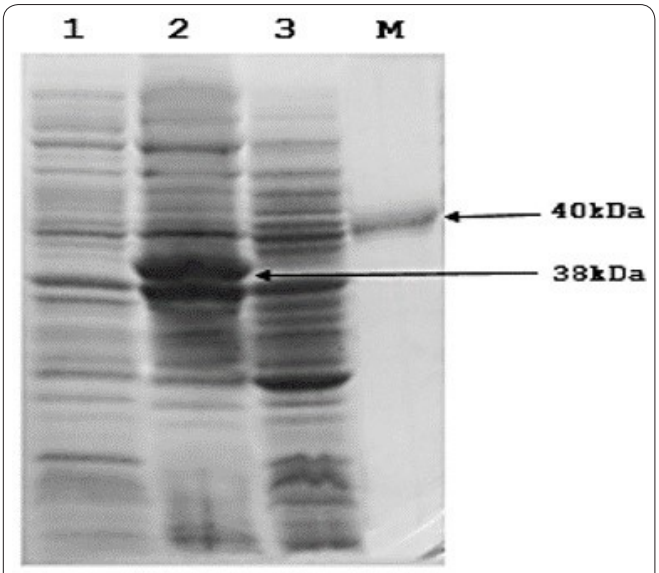

Figure 4. 12% SDS-PAGE gel showing expression in *E. coli* BL21 DE3 harboring PE38KDEL/pET. Lane 1: Uninduced pET22b, Lane 2: PE38KDEL/pET induced (total cell lysate), Lane 3: empty pET22b (+) induced, Lane M: Protein Marker ovalbumin (40kDa).

mobility of 38 kDa in Lane 4 confirmed that the recombinant protein was successfully produced.

To further investigate the cellular localization of the expressed protein, the cells grown in the manner stated above were harvested, centrifuged and resuspended in 50 mM Tris-Cl buffered at pH 8.0. After the disruption of cells by sonication, the supernatant corresponding to soluble fraction and the pellet representing the insoluble fraction were recovered in separate tubes by centrifugation. These cell fractions were then analyzed on 12 % SDS-PAGE (Figure 5). The protein was being expressed in both soluble and insoluble forms however the band of 38 kDa was slightly superior in supernatant samples indicating that more soluble protein was being produced as compared to insoluble.

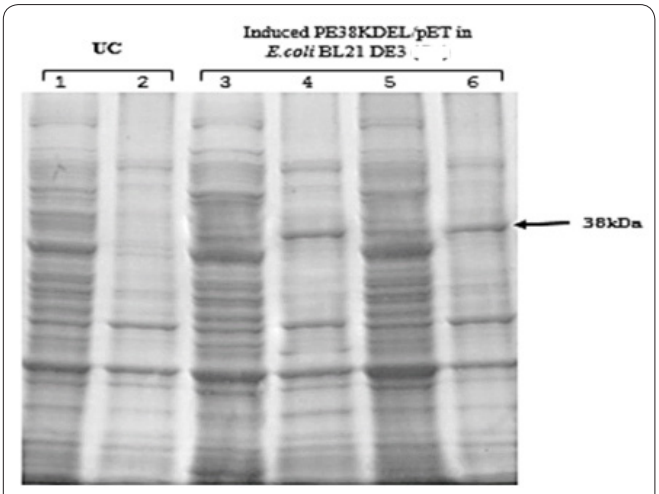

Figure 5. 12% SDS-PAGE gel showing expression in soluble and insoluble fractions of cell lysate of *E. coli* BL21 DE3 harboring PE33KDEL/pET constructs induced at 0.5 mM IPTG and 20 mM lactose concentrations. The arrow labelled as 38 kDa indicates the predicted proteins (PE38KDEL). Lane description: Lane 1: Uninduced pET22b (insoluble fraction/pellet), Lane 2: Uninduced pET22b (soluble fraction/supernatant) Lane 3: 0.5 mM IPTG (soluble fraction), Lane 4: 0.5 mM IPTG (insoluble fraction), Lane 5: 20.0 mM lactose (soluble fraction), Lane 6: 20.0 mM lactose (insoluble fraction).

Discussion

In the recent years, *Pseudomonas exotoxin* A, a highly potent protein, owing to its modular structure could be tamed into a remarkable therapeutic agent for targeted cancer therapy. PE is one of the most thoroughly researched toxin and evidently accessible to express in *E. coli* (28-34). Two most important investigations have led to the exploitation of PE in immunotoxins construction designs (12, 13). The first one is related to the interpretation of the crystal structure of PE, revealing that the toxin molecule consisted of three major structural domains (12). The second investigation demonstrated that all three domains performed their functions as independent modules (13). The recently developed lysosomal resistant form of PE based immunotoxins has three novel attributes. The removal of most of domain II portion excluded several sites prone to cleavage by lysozymes and formed a molecule that could be named as "LR" for lysosomal resistant (17). They are of small size, have reduced immunogenicity and better resistance to cleavage by lysozymes (16). Moreover, creation of the smaller PE versions can stabilize the toxin inside the cellular environment and preclude its disintegration in lysosomes. The protein expressed in this investigation was devoid of certain regions inessential to the programming of toxin protein for immunotoxins in view of literature (16, 20-22, 35, 36). Moreover, as an example of AB toxins the cytotoxic domain A of PE could remain stable and fully actively independent of receptor binding domain B (37). The expressed protein was termed PE38KDEL since it had 'KDEL' endoplasmic reticulum retention signal added at its carboxyl terminus. Seetharam *et al.* (38) investigated effect of changing the carboxyl sequence of PE from REDLK to the specific endoplasmic reticulum retention sequence, KDEL, **or** to KDEL repeated thrice $(KDEL)_3$. It was shown that PE molecule ending with KDEL or KDEL repeats were more effective in killing cells than the PE and its derivatives containing the REDLK at its end.

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Author's contribution

Hina Qaiser is the principle author who, in coordination with Saima Iftikhar, carried out all the experimental work and wrote this manuscript. Adeen Farooq also helped in research related work. Farheen Aslam critically reviewed the manuscript.

Interest Conflict: The authors declare that they have no competing interests.

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