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Designing a light-activated recombinant alpha hemolysin for colorectal cancer targeting

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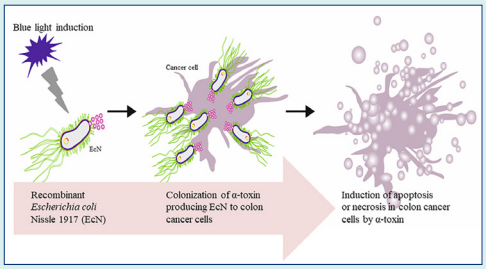
Abstract



Introduction: Colorectal cancer (CRC) is one of the main health burden worldwide, which can cause major economic and physiological problems along with relatively high rate of mortality. It is important to develop new methods for the localized delivery of recombinant protein therapeutics, in large part due to the failure of conventional therapies in most cases. Since *E. coli* Nissle 1917 (EcN) does not produce any virulence factors, here we used these bacteria with the light-activated promoter system to deliver therapeutic agents in the desired location and time.

Methods: In this study, *Staphylococcus aureus* alpha hemolysin (SAH), after codon usage optimization, was cloned into blue light activating vector (pDawn) and transferred to EcN strain. Then, the functionality and cytotoxicity of secreted alpha hemolysin was evaluated in the SW480 colon cancer cell line by using different experiments, including blood agar test, flow cytometry analysis, and DAPI staining.

Results: Our findings revealed that EcN can produce functional SAH under the blue light irradiation against SW480 cancer cells. Moreover, cytotoxicity assays confirmed the dose- and time-dependent toxicity of this payload (SAH) against SW480 cancer cells.

Conclusion: Based on our results, EcN is proposed as an appropriate light-activated vehicle for delivery of anticancer agents to the target cancer cells/tissues.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancers, and the fourth-highest cause of oncological deaths, therefore representing a major public health problem.[1,2] Most of conventional treatments such as chemotherapy, radiotherapy, and surgery usually affect only proliferating cells, and often cannot penetrate the deepest part of the tissue. These limitations might enhance the chance of recurrence, metastasis, and mortality.[3] Bacteria can be exploited to deliver and/or produce therapeutic molecules *in situ* in the desired time and location. Based on such features, the use of bacterial therapy might improve the treatment efficacy significantly.[4,5] Of note, the use genetically-modified bacteria to deliver therapeutic macromolecules under a controllable manner can offer some advantages over the classic treatment methods. As a result, various promoter systems have been established for the controlled expression of genes in bacterial systems by different chemical inducers such as light.[5,6] The main advantage of light, in comparison with chemical inducers, is the ability of light to turn off the expression by eliminating the light source.[7] Over the past years , several types of natural and engineered bacterial species have been developed and used in cancer treatment, including *Clostridium*, *Bifidobacterium*, *Salmonella*, *Bacillus*, *Listeria* and *Escherichia coli*.[8-10] The *E. coli* Nissle 1917 (EcN) bacteria has widely been used to treat gastrointestinal (GI) disorders such as acute diarrhea, inflammatory bowel disease (IBD), and others,[11] in large part because of its highest tumor-targeting ability among the *E. coli* strains.[12]

Ideally, protein payload should efficiently be released

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on-demand, penetrate tumor tissue, and effectively kill cancer cells.[13] The secreted protein must be functional after the secretion at the target site. In the case of solid tumors, it should also be noted that many bacterial species can aggregate in the extracellular space of tumors.[14] Various types of payloads have recently been used in bacterial therapy of tumors, including prodrug cleaving enzymes, cytokines, antigens and bacterial toxins.[15-18] Of these, the toxin, SAH, is a 34 kDa water-soluble and pore-forming hemolytic exotoxin that can cause membrane damage to many types of mammalian cells and induce apoptosis in cells.[19,20] Given that the majority of pore-forming proteins acts on the cells externally and do not require endocytosis, they are attractive candidate for the targeted therapy of tumor.[21] Based on these properties, in this study, EcN was selected as the carrier to deliver SAH to colon cancer SW480 cells. Thus, in this study, we hypothesized that the use of safe probiotic bacteria such as EcN, as a robust drug delivery system (DDS) with a triggereable drug release potential, might result in an improved cancer treatment.

Materials and Methods

Materials

SW480 cells were acquired from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). RPMI-1640 media, fetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK). Restriction enzymes *(Bam H*1 and *Xho*1) and *T4* DNA ligase were purchased from Thermo Fisher Scientific, (Waltham, MA, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from eBioscience (Waltham, MA, USA). Kanamycin and DAPI (4, 6-diamidino- 2-phenylindole) were attained from Sigma-Aldrich Co. (Poole, UK). Plasmid DNA extraction kit and DNA gel extraction kit were obtained from Qiagen (Hilden, Germany). The pDawn plasmid was purchased from Addgene (Watertown, MA, USA).

Plasmid and strain

The pDawn plasmid was used for the expression of recombinant proteins in response to the blue light induction.[22] Kanamycin with the concentration of 50 µg/mL was used to maintain the strains transformed with the pDawn-SAH. The *E. coli* Nissle 1917 (serotype O6:K5:H1) was obtained from Mutaflor® (Herdecke, Germany) and used after the modification. The SW480 cells were normally cultured with a seeding density of 2.0×10^4 cells/cm^2 and kept at 37°C and 5% CO_2 in a humidified atmosphere.

Cloning of alpha hemolysin

Plasmid DNA extraction was carried out using Qiagen Mini Kit (Hilden, Germany). The alpha hemolysin (*hia* gene) from *S. aureus* strain MW2 was codon optimized and synthesized with the appropriate restriction site for *BamH*I and *Xho*I restriction enzymes on the synthesized gene (GenScript, Singapore) for the expression in *E. coli*. Then, synthesized sequence was subcloned into pDawn plasmid (pDawn-SAH) using *BamH*I and *Xho*I restriction enzymes according to the protocols in published literature.[23] Afterward, the recombinant vector was transformed into the competent EcN using the heat shock method.[24] Briefly, 5 µL of DNA was added to 50 µL of the competent cells and gently mixed for a few times. Afterward, the competent cell/DNA mixture was incubated on ice for 20-30 minutes and located at 42°C in water bath for 45 seconds and placed back in ice for 2 minutes. The resultant bacteria were grown in LB media without antibiotic for 30 minutes in 37°C shaking incubator. Finally, some of the transformant bacteria were plated into LB agar media containing appropriate antibiotic. Consequently, the purified products were sequenced by Macrogene Company (Macrogene, Seoul, Korea).

Blood agar test

Overnight cultures of EcN transformed with pDawn-SAH were diluted 1:10^7 in LB and plated on blood base agar plates mixed with 5% sheep erythrocytes. After 12 hours of growth in the dark condition at 37°C for colony formation, plates were transferred to either light (blue~480 nm, 37.1 µW/cm^2) or dark conditions at 25°C for 24 hours prior to imaging.[25]

Anticancer potential of SAH

Overnight cultures of EcN transformed with pDawn-SAH were diluted and allowed to grow to an OD_{600} of ~1.0 and then induced with blue light (~480 nm and 37.1 µW/ cm^2) or dark conditions at 25°C for 24 hours. Then supernatant of induced and non-induced cultures were sterilized and subjected to next experimental procedures. Cytotoxicity assay was evaluated using different methods, including trypan blue assay, flow cytometry analysis, and DAPI staining.

Cell viability test

The cell viability assay was carried out using trypan blue exclusion test. Briefly, SW480 cells were seeded in a 24 well plate and incubated for 24 hours at 37°C with 5% CO_2. Subsequently, the cells were treated with two different concentrations (0.5% and 1% (v/v)) of various supernatants and incubated up to 24 hours. Treated cells were washed with PBS (3x) and detached with trypsin. Then, 100 µL trypan blue was added to the cell suspension and maintained for 5 minutes at room temperature. Finally, the viable and dead cells were counted.[26]

Flow cytometry analysis

The flow cytometry analysis was used to detect apoptotic and necrotic population of the treated SW480 cells. Manufacturer's protocol was used to prepare samples. Shortly, cells were treated with supernatant of induced and non-induced cultures for 6 hours (OD_{600}: 1.0) and

centrifuged at 111 ×g for 5 minutes and cell pellets were washed with PBS (3x). Subsequently, the cells were stained with annexin V-FITC for 15 minutes, and incubated at room temperature in the dark. Finally, the cells were transferred to the PI binding buffer and prepared for the measurement of apoptosis. The Cell Quest software (Becton Dickinson, San Jose, USA) was used for the analysis of data.

DAPI staining

The SW480 cells were seeded in 6-well plates, allowed to reach 70% confluency, and treated with sterile supernatant of 6-hour induced and non-induced cultures. Then, 4% paraformaldehyde was used for the fixation of the cells for 10 minutes and washed with PBS (3x), then permeabilized with 0.1% Triton X-100 for 10 minutes. Finally, 4′, 6-diamidino-2- phenylindole (DAPI) was used to stain the treated cells for 5 minutes. The IX81 inverted fluorescent microscope (Olympus Corp., Tokyo, Japan) equipped with an Olympus DP72 digital camera was used to evaluate the morphology of the cells.[27]

Results

Sub-cloning of alpha hemolysin

The pUC57 plasmid containing synthesized SAH and also pDawn plasmid were simultaneously subjected to *BamHI* and *XhoI* restriction enzymes and digested products were run in 2% gel electrophoresis. The digested product of *hia* gene was purified using DNA gel extraction kit and ligated with the restricted pDawn vector using *T4* DNA ligase enzyme as shown in Fig. 1.

Blood agar assessment

The blood agar test was performed for the verification of the functional α-hemolysin production by the transformed EcN. For this purpose, the kanamycin-resistant transformants were cultured onto blood agar plates. Clear zones of lysis were observed around the colonies that were transformed with pDawn-SAH under the blue-light induction, while no clear zones of lysis were achieved in dark condition (Fig. 2).

Cell proliferation assay

The trypan blue staining is a rapid and convenient assay that can analyze the number of live (unstained) and dead (blue) cells by means of light microscope.[26] In this study, fresh supernatants of EcN, EcN-dark and EcN-blue light were subjected to SW480 cells in various concentrations of 0.5% and 1 % (v/v). As shown in Fig. 3, the rate of dead cells treated with 0.5% (v/v) of supernatant were more than 50% in the EcN-blue light and about 20% in EcN and EcN-dark in 10 hours, while in the cells treated with

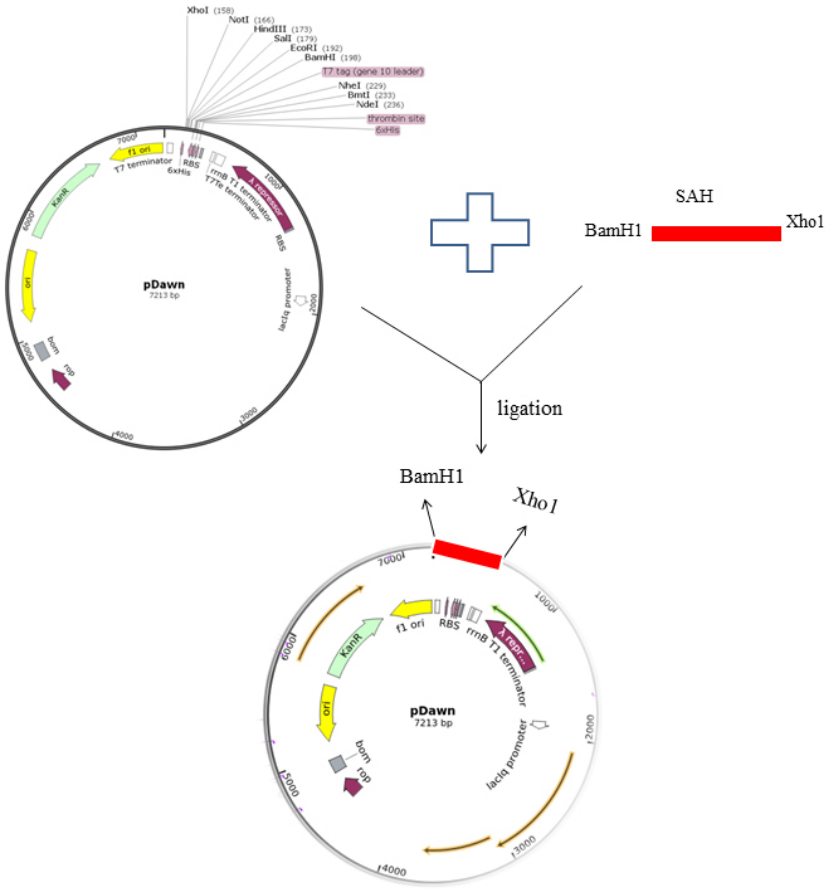

Fig. 1. Schematic illustration of SAH sub-cloning in pDawn plasmid with restriction enzymes.

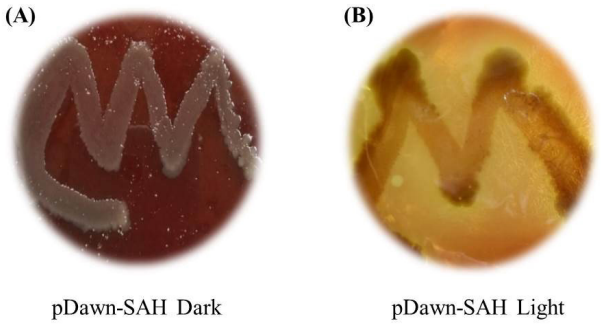

Fig. 2. Blood agar tests with the pDawn-SAH transformed in EcN. (A) No clear zone in non-induced culture in dark. (B) Clear zone around the colonies indicating the blue light-dependent expression of α-hemolysin.

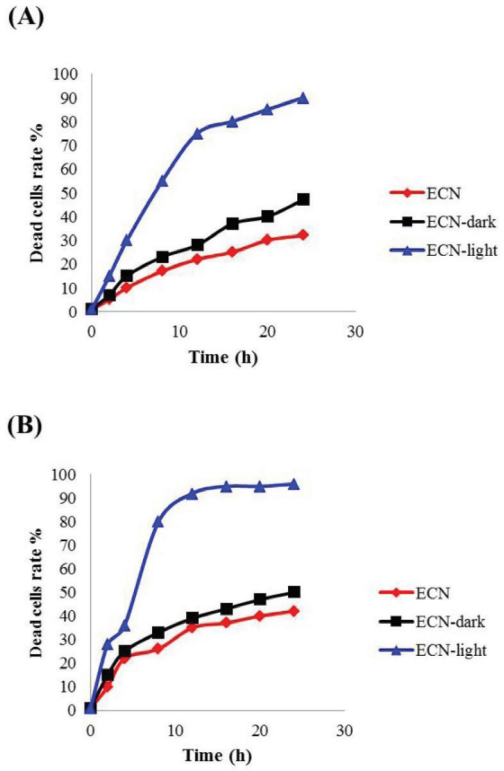

Fig. 3. Effect of different supernatants on the SW80 cells. Fresh supernatants of EcN (control without vector), EcN-dark (control with vector) and EcN-blue light (EcN including pDawn-SAH induced with blue light) were administered to SW80 cells in a final concentration of (A) 0.5% and (B)1% (v), (n=3).

1% (v/v) these ratios were more than 80% for EcN-blue light and about 30% for EcN and EcN-dark in 10 hours. Therefore, these results indicated that the rate of dead cells including apoptotic and necrotic cells were increased by increasing the time and concentration of toxin.

Annexin V apoptosis detection by flow cytometry

Annexin V based flow cytometry assay was carried out for the separation of the necrotic and apoptotic cells from the normal cells. Annexin V binds to phosphatidylserine (PS) of the plasma membrane that externalized after apoptosis.[28] Compared to the untreated control cells (Fig. 4A), about 26.34% apoptosis and 1.20% necrosis were observed in the SW480 cells treated with sterile supernatant of non-induced (Fig. 4B), while 36.08% apoptosis and 48.41% necrosis were observed in the SW480 cells treated with the sterile supernatant of induced cells (Fig. 4C).

DAPI staining and cell apoptosis

The analysis of the morphological changes was performed using DAPI staining after 6 hours of the treatment with the sterile supernatant of non-induced and induced cultures (Fig. 5). As compared to the control cells (Fig. 5A), no morphological changes were observable in the treated non-induced cells (Fig. 5B), while a significant fragmentation was observed in the nucleus of the light-induced cells (Fig. 5C) .

Discussion

In this study, we capitalized on the EcN as a vehicle to deliver biologically active SAH to the CRC cells. SAH is a pore forming protein whose cytolytic and/or cytotoxic activity against many mammalian cell types has been shown in several studies.[20,29] It can cause membrane damage in the multiple cell types without need to endocytosis that makes it an effective treatment in multidrug resistant tumors.[30] The use of live engineered bacteria, as nonpathogenic safe strains with the controllable stimuli, offer the localized and continuous drug delivery potential to prevent malignant cells from restoring their membrane by persistent representation of the toxin.[31] Different light-inducible gene expression strategies have recently been reported in mammalian cells with some advantages such as the tunability potential and the spatiotemporal impacts.[32,33] This study was divided into two parts. First, we cloned and evaluated the functional expression of SAH protein in the presence of blue light (induced) and without light (non-induced) using the pDawn plasmid (Fig. 1) as a light-activated expression system for light regulated expression of recombinant proteins in *E. coli*. Second, we looked at the anticancer effect of produced SAH against SW480 cells by different techniques. Our results indicated that EcN can successfully express and secrete SAH into the culture under the blue light induction. This finding was in agreement with a recent study that indicated effective expression of cytolysin A by EcN under blue light induction.[34]

Of note, the main objective in the construction of gene products is the assessment of the functionality of products. Because some modifications made in gene structure like His-tag addition to the N-terminal or C-terminal may show somewhat conflict with the formation of native and functional protein structure. Blood agar test is a simple and cost-effective experiment that has been done in many studies for the evaluation of cloned hemolysin.[19,25]

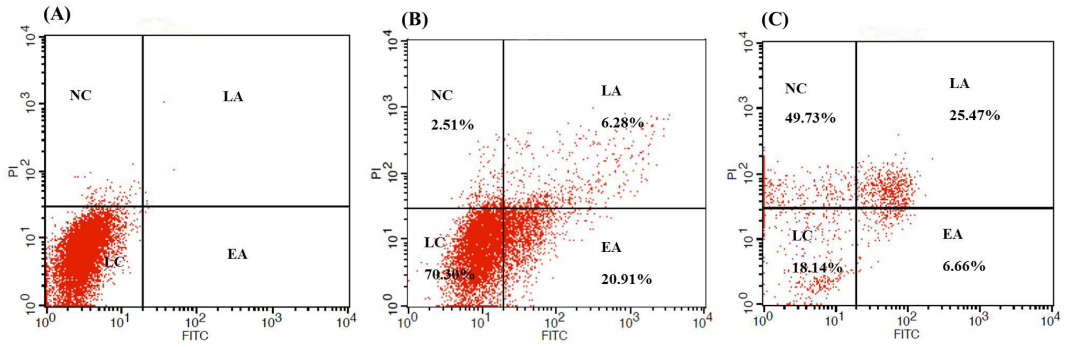

Fig. 4. The flow cytometry analysis of the FITC-AnnexinV / PI showing apoptotic and necrotic cells rate in the unstain SW480 cells (A) and cells treated with sterile supernatant of non-induced panel (B) and induced (C) cultures for 6 hours. **NC**: Necrotic cells; **EA**: Early apoptosis; **LA**: Late apoptosis; **LC**: Living cells.

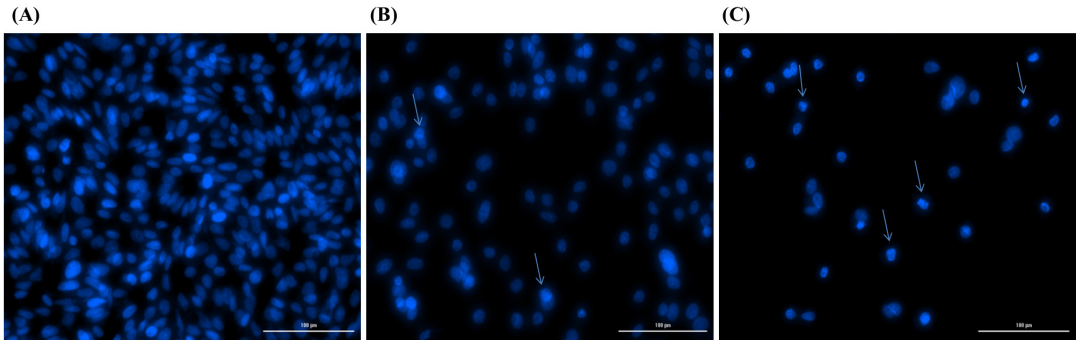

Fig. 5. Representative photograph showing DAPI staining of untreated control SW480 cells (A) and cells treated with sterile supernatant of non-induced (B) and induced (C) cultures for 6 hours. Morphological changes such as nuclear fragmentation and chromatin condensation reveals apoptosis in blue light induced treated cells (C).

Blood agar test confirmed that the secreted SAH protein is fully functional and does not need any additional modification(s). We also investigate the cytotoxicity of produced toxin on the SW480 cells. It should be pointed out that rypan blue is used in cell viability test, in which live cells have undamaged plasma membranes that can eliminate different chemicals like trypan blue, while dead cells have broken plasma membranes and cannot exclude trypan blue.[26] Since pore forming toxins cause host cell membrane damage, it is possible that the created pores by toxin may allow trypan blue to enter the host cell.[19,35] The cell viability assay accomplished in this study confirmed that affectivity of SAH is largely dependent on the exposure time of toxin (Fig. 2). Such a finding was in good agreement with other experiments accomplished with bacterial toxins.[35] Likewise, based on the literature, effect of SAH on the cells depends on the amount of toxin and cell types. For example, the high concentration of toxin mostly yields necrosis, while the low concentration of toxin might induce apoptosis or inflammatory response in the target cells.[36-38] Our results were found to be in agreements with such findings. As confirmed by the flow cytometry results, supernatant of non-induced cultures could cause apoptosis (Fig. 3B), while supernatant of light induced cultures cause necrosis more than apoptosis in SW480 cells (Fig. 3C). Furthermore, the presence of apoptosis in non-induced cultures can elicit this suggestion that EcN alone can induce apoptosis to a certain degree or EcN express SAH to some extent without the blue light induction. This finding was further confirmed by DAPI staining method. A few number of apoptotic cells were observed in non-induced treatments compared to the control cells (Fig. 5), and clear apoptosis was observed in light-induced treatment (Fig. 5A). Mechanistically, SAH is speculated to directly elicit membrane damage in the cell membrane and perhaps interfere with the function of cell surface transporters too (Fig. 6). Notably, anticancer properties of probiotic bacteria can occur through the regulation of different stages of signaling pathways and hence the induction of apoptosis as reported in many studies.[39-41] Besides, some clinical studies have reported the *in situ* expression or delivery of tumor-specific proteins, antibodies, cytokines, and prodrugs. It seems they are considered to be a relatively applicable delivey system while the safety and effective tumor-colonization feature of these bacteria need to be fully addressed.[42,43] Taken all, safe non-pathogenic probiotic strains like EcN could offer some benefits, including simplicity in genetic manipulation, and lack of toxicity to the normal cells. Based on these results, EcN could be used as a competent drug delivery vehicle for the localized delivery of anticancer agents.

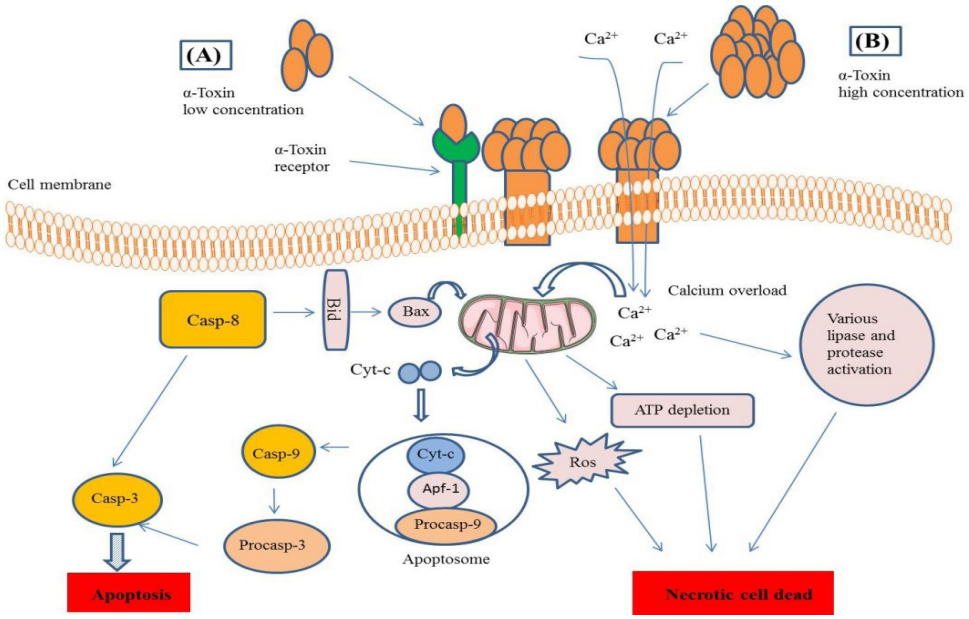

Fig. 6. Schematic representation of α- toxin pore formation mechanism and cell death pathways. (A) The α- toxin at low concentration usually attaches to specific receptor (ADAM10) to oligomerize and form pore and trigger intrinsic or caspase related apoptotic pathway. (B) At the high concentration, it makes membrane pore without need to the specific receptors and trigger apoptotic or necrotic cell dead signaling pathways through Ca^{2+} influx and perturbation of Ca^{2+} or other ions hemostasis.

Conclusion

Since the cancer is a complex disease with different mechanisms of creation, chemotherapy or other treatment approaches are not enough to cure all parts of the cancer alone.[44,45] Therefore, combination of conventional therapies with new techniques seems to be more profitable. Bacterial based therapy specially using nonpathogenic or safe strain displays more promises due to possible capability of bacteria to deliver active therapeutics into the cancerous tissue. Therefore we aimed to investigate anticancer properties of SAH delivered in the nonpathogenic EcN by induction of blue light *in vitro*. Based upon our results, EcN is a good bacterial system to deliver active therapeutic into the tumoral region by light controlled manner as well as SAH which has strong cytotoxicity against SW480 colon cancer cells. However, additional studies with various cell lines and also *in vivo* examinations are needed for acquiring precise information for the clinical application.

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Ethical statement

None to be declared.

Competing interests

The authors declare that YO is the EIC of the journal and had no influnce on the peer-review process, which was conducted carefully based on COPE guideline.

Authors' contribution

SA, AE and YO developed the concept of the study. SA performed the experiments. AB contributed to the design of research and experiment. SA drafted the manuscript. YO finalized the manuscript. All the coauthors approved the submission of the manuscript.

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