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(54) Title: FUSION PROTEIN FOR CANCER THERAPY

(57) Abstract: The present invention discloses a fusion protein for cancer therapy. The invention discloses a fusion protein economically produced using *Escherichiacoli* as an expression host. In the present invention, the target specific cell penetration is achieved by a fusion of CPPs. The present invention also discloses the production and purification of the fusion proteins for downstream applications.



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FUSION PROTEIN FOR CANCER THERAPY

FIELD OF THE INVENTION

The present invention relates to a fusion protein for cancer therapy. The invention provides an affordable approach towards achieving cell-penetrating peptide (CPP) mediated targeted delivery of a photosensitizer protein and light assisted killing of the cancer cells. The fusion protein is economically produced using *Escherichia coli* as an expression host. The target specific cell penetration is achieved by fusion of CPPs. The key idea is to house the photosensitizer protein specifically inside cancer cells and then expose it to a light of a desired wavelength so as to achieve cancer cell killing by production of reactive oxygen species (ROS). The damage to normal cells is minimized by two step selection procedure i.e. first level of specificity is achieved by targeted delivery of the fusion protein to the target site and second level of specificity is achieved by the selective killing of the target cancer cells by exposing to a light of desired wavelength. The invention describes the production and purification of the fusion proteins for downstream applications.

BACKGROUND OF THE INVENTION

Cancer has been a cause of concern for health care givers since long. Despite the serious efforts that have been put towards meeting the goal of combatting this problem, the ultimate goal remains yet to be achieved. The World Health Organization still attributes 1 in 6 deaths to cancer (WHO *cancer key facts*). Anti-cancer research drives the biomedical field to an extent that a plethora of options are available for various cancer types. Depending on the extent and the spread of the disease, the treatment modality is chosen. The key question still remains, are we even close enough in deciphering an efficient treatment option that could fight the disease without affecting the non-cancerous tissue?

Along with the available chemotherapies, the recent advancement in the field of immune therapy has given excellent results in fighting against cancer cells. This therapy leads to up-regulating the immune system to kill cancer cells (*Cancer.gov; Immunotherapy to*

treat cancer). Many other specific and efficient therapy options apart from the chemotherapy and radiotherapy have come up in recent years, as in, targeted therapy, hormonal therapy, stem cell transplant and precision therapy (*Cancer.gov; types of cancer treatment*).

- 5 The prohibitory cost of therapies, poor success rate, and undesired side effects, all contribute in making cancer still an unmet medical need. The major concern of targeting only cancer cells while maintaining the efficiency of the therapy is yet to be achieved.

Photodynamic Therapy (PDT) is the most upcoming and one of the most promising of the new options. Although PDT was first of the drug-device combination to have gained
10 the United States Food and Drug Administration (US-FDA) approval, its potential remains to be explored completely (*Agostinis et.al. 2011*). The PDT essentially requires a photosensitizer, a reactive product: singlet oxygen and a light dose along with its fluence (*Agostinis et.al. 2011*). So, in simple words, a photosensitizer is administered followed by illuminating it with a light of the appropriate wavelength. This transforms
15 the drug from its ground state to an excited state, coming back from which, the devastating singlet oxygen species are produced, leading to the killing of the cell in which the drug or the photosensitizer resides (*Triesscheijn et al. 2006*). The effect and efficiency of this treatment, however, depend on a few factors like the photosensitizer type, the drug concentration, and its localization inside the cancer cell, light source fluence, its dose,
20 and the singlet oxygen generation (*Triesscheijn et al. 2006*). Ideally, a photosensitizer must have a significantly good uptake into the tumor with easy clearance and a strong absorption peak at its specific wavelength. Porfimer Sodium and *meta*-tetrakis (3-hydroxyphenyl) chlorin(mTHPC) are examples to name a few (*Triesscheijn et al. 2006*).

Then, there are genetically encoded photosensitizers i.e. fluorescent proteins. They
25 generally comprise a chromophore region that produces the reactive oxygen species on light irradiation, which kills the cells in which they reside. This is referred to as the Chromophore-Assisted Light Inactivation (CALI) (*Bulina et al. 2006*). Among the genetically encoded ones, Green Fluorescent Protein (GFP) is a prominent one but is an inefficient photosensitizer as the protein shell that screens its chromophore prevent
30 efficient ROS generation (*Surrey et al. 1998*). Bulina et al, screened various homologs of GFP in search of an efficient photosensitizer and could identify Killer red (KR) as a

chromoprotein from *Anthomedusae* sp. DC-2005 with a strong phototoxic effect (*Bulina et al. 2006*). They characterized the phototoxic profile of this protein and identified that it depicts best cell killing activity in presence of green light (540 – 580nm) and killed more than 90% of *Escherichia coli* (*E. coli*) in around 10 minutes on irradiation. They also showed that KR could induce chromophore assisted light inactivation (CALI) much efficiently than GFP. Post their findings, many groups worked in various ways to bring out the best from this protein for cell killing (*Bulina et al. 2006; Liao et.al. 2013*). The scientists demonstrated efficient destruction of cells in which it resides on being illuminated with a green light.

Evrogen in Russia has come up with several commercial KR expression/source vectors also (*Evrogen.com*). They have worked extensively on the protein and have explored its photosensitizer properties along with its mitochondrial and cell membrane specific variants.

Based on this background, it can be inferred that there is a potential therapy at hand, which needs to be explored to its best for the most sought after areas of research. Amidst the available options, this seems like a candidate that could have a promising effect. It is however evident that the gene therapy of a protein for cancer therapy will be an expensive take. The available gene therapy approaches will undoubtedly meet the desired effect but will not be an affordable option. The disease, on the other hand, does not discriminate based on the financial status of the person. So for those from the lower-income groups might go unaffected of its benefits.

It has been hypothesized that if this protein KR is expressed outside in cheaper living systems like bacteria, *E coli* for instance, and expressed along with sequences at one of its terminals that would probably assist in its internalization inside the tumor cells, the outcome could be a whole lot cheaper version of a similarly efficient therapy that could benefit those affected on a larger platform.

The specific peptides that help carry larger loads, drugs or proteins inside cells are referred to as cell-penetrating peptides (CPPs) (*Guidotti et al. 2017*). It is these CPPs that help traverse stringent barriers and assist in reaching areas that most molecules cannot easily. So achieving internalization with the help of CPPs seems to solve many problems and achieve high potency and applicability of difficult therapies (*Kristensen et al. 2016*).

Now, since these photosensitizers are activated by certain wavelengths of light, the light source must be able to penetrate enough to reach the target cancer cell with the photosensitizer protein. In 2017, Liang et al., developed Upconversion nanoparticles to help the photosensitizers like KR to work in deep-seated tumors as well. These bio-nano hybrids can convert the deep penetrating near-infrared rays to light in the visible spectrum, which in turn will excite the photosensitizer, covalently attached to it. The only drawback of this strategy of delivering the photosensitizer along with Upconversion nanoparticles is that the Upconversion nanoparticles bound photosensitizer internalized the cancer cells in 12 hours and that this strategy does not provide any specific tumor targeting.

Some prior arts in this area provide insights towards the applications and types of photosensitizers (*WO2011/096501 A1*; *2016/9296797 B2*; *EP 2013/2606360 A1*; *2013/7910714 B2*; *2017/0051030 A1*; *CN 2018/107936091*). Although these prior arts talk about delivering the photosensitizers of the like of KR and its functional variants, none of these has been able to bring out the approach targeted in the invention. Therefore, there is a need in the art for improved photodynamic therapy for cancer cells using a photosensitizer along with CPP as a single fusion protein having efficient internalization.

OBJECT OF THE INVENTION

The main objective of the present invention is to provide a fusion protein for cancer therapy.

Another objective of the present invention is to provide a novel fusion protein containing a CPP (cell-penetrating peptide) tagged photosensitizer protein KR.

Yet another objective of the invention is to provide an *E coli* system for expression of the novel CPP tagged photosensitizer fusion protein c. The fusion protein is purified from a soluble fraction.

Another object of the invention is to link the fusion protein to the Upconversion nanoparticles to increase effective wavelength spectra in the red or infrared region to improve tissue penetration.

Still another objective of the present invention is to enhance the spectra of wavelengths

that can excite the photosensitizer protein internalized into even deep-seated tumors.

SUMMARY OF THE INVENTION

An aspect of the present invention provides a fusion protein for cancer therapy consisting
5 of:

- a. a photosensitizer protein; and
- b. a cell-penetrating peptide.

Another aspect of the present invention provides a fusion protein for cancer therapy, wherein the photosensitizer protein is Killer Red (KR).

- 10 Yet another aspect of the present invention provides a fusion protein for cancer therapy, wherein a photosensitizer protein is fused with a cell penetrating peptide to yield a fusion protein having the amino acid sequence selected from the group consisting of SEQ ID NOs. 2 and 4-17.

- 15 Still another aspect of the present invention provides a plasmid DNA construct comprising a gene encoding the fusion protein having the polynucleotide sequence selected from the group consisting of SEQ ID NOs.: 24 and 26-39.

Yet another aspect of the present invention provides a plasmid DNA construct wherein said construct is expressed in *Escherichia coli* cells.

- 20 Another aspect of the present invention provides a method for targeting the fusion protein into a cell comprising the steps of:

- a. incubating the fusion protein with a cell at 37 °C for 30 minutes – 12 hours; and
- b. activating the fusion protein by exposure to visible light, preferably green light.

- 25 Yet another aspect of the present invention provides a method for targeting the fusion protein into a cell, wherein the cell is selected from a group consisting of HeLa cell, Hep3B cell, prostate cancer cell, MCF-7 cell, glioblastoma cell, breast cancer cell, hepatocellular carcinoma cell, normal hepatocyte cell, HUVEC cell, HMVECd cell,

A375M cell, MCF-7 cell, MDA-MB-231 cell, HT-29 cell, Jurkat cell, CEM cell, peripheral blood mononuclear cell, DU145 cell, A875 cell, M14 cell, A549 cell, ZR-75-30 cell, Raji cell, NB4, K562 cell, lung cancer cell, gastric cancer cell, neuroblastoma cell, pancreatic ductal cancer cell, lymphatic cancer cell, and lung cancer cell.

- 5 Another aspect of the present invention provides the fusion protein for use in attaching to upconversion nanoparticles.

BRIEF DESCRIPTION OF DRAWINGS

- 10 **Figure 1:** A Schematic of the construct designed. The photosensitizer protein, of the likes of KillerRed along with a CPP. A-D showing fusion of CPP at different possible locations i.e. N-terminal, C-terminal, or both N-and C-terminal or loop regions on the photosensitizer protein.

This drawing shows the possible and lucrative sites or loops, 3, 5, 6, 7, 8 and 9, in the
15 construct where the CPP of interest could also be incorporated so as to enhance specificity and targeting efficiency.

Figure 2: Purification of the fusion protein. **A.** Schematic representation of fusion construct designed. **B.** Purification of the fusion protein (KR-PEN) using Ni-NTA chromatography. M, Marker; P, Pellet after sonication and centrifugation; F, Flow
20 through; W, wash fraction; E1 to E4 elution fractions with increasing concentration of imidazole. **C.** A representative analytical gel filtration profile of the fusion protein showing major peak corresponding to the protein of interest corresponding to the oligomeric state of the protein in solution. **D.** SDS-PAGE profile showing purified fusion constructs having different CPPs fused to the C-terminal of the photosensitizer protein.

25 **Figure 3:** Representative vector maps for the recombinant fusion proteins. **A.** pET28c vector with killer red gene. **B.** pETDuet-1 vector with KR-PEN fusion gene and **C.** pETDuet-1 vector with GFP_PEN fusion gene.

Figure 4: Cell internalization experiments, FACS & Confocal in HeLa cells.

Figure 5: Photodynamic killing of the cells internalizing the photosensitizer protein, time
30 as a variable factor.

Figure 6: The fusion protein covalently linked to the Upconversion nanoparticles, enabling conversion of deep penetrating infrared light to visible light that activates the photosensitizer to kill the cell in which it homes.

5 ABBREVIATIONS

	CPP	Cell penetrating peptide
	CALI	Chromophore-Assisted Light Inactivation
	PCR	Polymerase chain reaction
	ROS	Reactive oxygen species
10	PDT	Photodynamic therapy
	GFP	Green fluorescent protein
	FACS	Flow assisted cell sorting
	FITC	Fluorescein isothiocyanate
	DAPI	4', 6-diamidino-2-phenylindole
15	DMEM	Dulbecco's Modified Eagle's Medium
	FBS	Fetal bovine serum
	PBS	Phosphate Buffer Saline
	NCCS	National Centre for Cell Science
	US-FDA	United States Food and Drug Administration
20	WHO	World Health Organization
	mTHPC	<i>meta</i> -tetrakis (3-hydroxyphenyl) chlorin
	<i>E. coli</i>	<i>Escherichia coli</i>
	LED	Light Emitting Diode
	HeLa cells	Henrietta Lacks cells

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed towards a novel fusion protein comprising a photosensitizer protein, and a CPP at one or more of its terminals to enable the fusion protein to internalize specific cells (Figure 1). The key idea behind designing the fusion protein was that photosensitizers when exposed or irradiated with light sources of specific wavelengths, get excited to different energy levels, and on return to their original energy levels, emit reactive oxygen species which are lethal for living cells, and cause cell death. This mechanism is exploited here, wherein the gene of a photosensitizer is tagged to a gene that codes for a CPP to express a fusion product of a CPP tagged photosensitizer protein, that is able to internalize inside the cancer cells and upon exposure to a specific light source, is able to kill these cancer cells.

The present invention is directed towards a fusion protein for cancer therapy consisting of:

- a. a photosensitizer protein; and
- b. a cell penetrating peptide.

In an embodiment of the present invention, there is provided a fusion protein, wherein the photosensitizer protein is Killer Red (KR).

In another embodiment of the present invention, there is provided a fusion protein, consisting of a photosensitizer protein fused with a cell penetrating peptide, to yield a fusion protein having the amino acid sequence selected from the group consisting of SEQ ID NOs. 2, and 4-17.

Another embodiment of the present invention provides a plasmid DNA construct comprising a gene encoding the fusion protein.

In the present invention, a novel plasmid DNA construct comprising the gene encoding a photosensitizer protein, along with a gene encoding a cell penetrating peptide is having the polynucleotide sequence selected from the group consisting of SEQ ID NOs 24 and 26 to 39.

The gene encoding the known photosensitizer protein and gene encoding the cell penetrating peptide, together, are inserted into a suitable vector, expressed efficiently in a bacterial host system to obtain the fusion protein.

5 In another embodiment of the present invention, there is provided a plasmid DNA constructs wherein said construct is expressed in *Escherichia coli* cells.

The present invention describes how CPP tagged version of a photosensitizer protein is purified from an *E. coli* expression system. In the present invention, the inventors have been able to purify this CPP tagged photosensitizer protein via the Ni-NTA column. The present invention describes a gel filtration of this Ni-NTA purified protein in a buffer
10 system suitable for cell culture studies.

There are certain portions of the C-terminal of the photosensitizer protein, which cause it to degrade, when truncated, led to a fusion protein with better stability and improved internalization inside HeLa cells. The fusion protein has been incubated with HeLa cancer cells to allow for internalization. (Figure 4). The present invention describes
15 through flow cytometry experiments, the time taken by the novel fusion protein to internalize into HeLa cells. The novel fusion protein, with an excitation wavelength of 540-545nm and an emission wavelength of 610 nm, has been observed in flow cytometry and acquired via the PE-Texas Red laser and the similar CPP tagged fluorescent protein through the FITC laser channel. Through this disclosure, it has been shown that of the
20 fusion protein of molecular weight 29.92 kDa (KR-PEN having amino acid sequence as set forth in SEQ ID NO. 2) internalizes inside HeLa cancer cells to a more than 90% in 4 hours. Further, it has been shown that the cell population internalizing the CPP protein remains intact and no cell death is observed with the CPP protein alone. Further, the novel fusion protein internalize inside HeLa cancer cells can be visualized via confocal
25 microscopy, wherein this protein is visible with the Texas Red filter. The present invention also shows that similar analogous CPP tagged fluorescent protein is visible under the confocal laser filter FITC (Fluorescein isothiocyanate based filter).

Still another embodiment of the present invention provides a method for targeting the fusion protein into a cell comprising the steps of:

30 a. incubating the fusion protein with a cell at 37 °C for 30 minutes – 12 hours; and

- b. activating the fusion protein by exposure to a visible light, preferably green light.

In yet another embodiment of the present invention, there is provided a method for targeting the fusion protein into a cell, wherein the cell is selected from a group consisting of HeLa cell, Hep3B cell, prostate cancer cell, MCF-7 cell, glioblastoma cell, breast cancer cell, hepatocellular carcinoma cell, normal hepatocyte cell, HUVEC cell, HMVECd cell, A375M cell, MCF-7 cell, MDA-MB-231 cell, HT-29 cell, Jurkat cell, CEM cell, peripheral blood mononuclear cell, DU145 cell, A875 cell, M14 cell, A549 cell, ZR-75-30 cell, Raji cell, NB4, K562 cell, lung cancer cell, gastric cancer cell, neuroblastoma cell, pancreatic ductal cancer cell, lymphatic cancer cell, and lung cancer cell.

When these HeLa cancer cells which have internalized the fusion protein, are exposed to a light source matching the excitation wavelength of the photosensitizer protein, cell death happens. The invention here shows the cell killing kinetics via confocal microscopy, over a period of time points starting from 10 minutes. The fusion protein residing inside the HeLa cancer cells is able to kill the cell completely by 45 minutes of light source exposure.

The present invention compares the cell killing ability of the fusion protein to a CPP tagged similar fluorescent protein, when both residing inside HeLa cells are exposed to the same light source for a specific time.

The novel fusion protein of the present invention, with a truncated C-terminal portion, has cell internalizing and killing properties when exposed to a light source of a wavelength similar to its excitation wavelength.

Another embodiment of the present invention provides the fusion protein for use in attaching to upconversion nanoparticles.

In an embodiment of the present invention, the fusion protein comprising the photosensitizer protein and the CPP for tumor targeting, will be bound covalently to an Upconversion nanoparticle, that would help the fusion protein internalized inside the deep seated tumors, as per the targeting, to be functional and efficient by being able to convert the deep penetrating infra-red radiations into visible light, of the desired wavelength (Figure 6).

The deeper tissue penetration if achieved by specific CPPs, will render the fusion protein non-functional as visible light of the desired wavelength is unable to cross tissues. On conjugating the novel fusion protein expressed in an *E. coli* expression system, with the Upconversion nanoparticles, as described by Liang et al. in their work in 2017, the overall
5 wavelength spectra of the product can be enhanced along with the targeting to specific tumor cells. The Upconversion nanoparticles will allow the conversion of the deep penetrating infra-red light to the visible spectra needed to excite the photosensitizer molecule to perform its cell killing function.

In the present invention, KR is fused with cell penetrating peptide(s), and expressed as a
10 recombinant protein using *E. coli* as an expression host. This fusion protein efficiently internalizes in the HeLa cells and causes effective killing upon irradiation with the suitable light source. The target specific internalization is primarily dictated by the CPP and targeted killing of cells is determined by region exposed to the suitable light source.

Expressing the photosensitizer along with the CPP as a single fusion protein with
15 efficient internalization and cell killing, holds immense potential to fill in the lacunae in the field of cancer therapeutics.

The fusion protein of the present invention is able to internalize inside HeLa cells. This internalized fusion protein is able to cause cell killing on being exposed to a light source of the wavelength similar to the protein's excitation wavelength. The cell killing activity
20 of the fusion protein is only in the presence of the light source and not otherwise. This specific tumor homing CPPs tagged to a photosensitizer protein leads to a targeted type of anti-cancer photodynamic therapy.

In the present invention, CPPs can be fused to the N-terminal, C-terminal or loop regions of the Photosensitizer. Further, the fusion protein of the present invention can be linked
25 to the Upconversion nanoparticles to increase effective wavelength spectra in the red or infrared region to improve tissue penetration.

In the present invention the fusion protein causes cell killing on HeLa cell lines on being irradiated with a green light source, observed over fixed time points of 10, 20, 30 and 45 minutes.

The present invention has been able to determine via confocal microscopy the cell killing efficiency of the fusion protein compared to a similarly CPP tagged analogous fluorescent protein and upon no or light source exposure to both the protein constructs.

In the present invention, it has been demonstrated successfully that the novel fusion
5 protein internalizes in >90 % HeLa cells in 4 hours, and post this, upon exposing it to a
suitable light source for 20-30 minutes, kills the cells in which it has internalized. Further,
the cells with fusion protein internalized remained intact in the absence of the light
source. Furthermore, the cells that internalized the CPP tagged GFP fluorescent protein,
has about 1000 fold less efficiency in producing ROS, did not die even in the presence
10 of the light source, confirming that neither CPP alone or GFP alone is harmful for cells.

EXAMPLES

The following example is given by way of illustration of the present invention and
therefore should not be construed to limit the scope of the present invention.

15

Example 1:

Method for preparing a fusion protein

The codon optimized synthetic gene of the photosensitizer, KR (source-*Anthomedusae*
sp. DC-2005 and GeneBank accession number AY969116) was procured from
20 GeneScript (USA). Gene encoding KR from pUC19 was digested with NdeI and XhoI
restriction enzymes and further cloned into pET28c (Novagen Inc. Madison, USA) with
an N-term 6x-His tag (amino acid sequence as set forth in SEQ ID NO. 1). The 6x-His
tag was specifically inserted for the ease of affinity purification. The different CPPs were
genetically fused to the 3' end of the KR gene using primers procured from Integrated
25 DNA Technologies (USA) and Sigma Aldrich (USA). The DNA constructs encoding the
fusion protein were cloned in pET28c and pETDuet-1 vectors and expressed in BL21
(DE3) *E. coli* strain (Figures 2A & 3). Both the plasmids and BL21 (DE3) strains were
procured from Novagen Inc. (Madison, USA cat # 69450-3). Now, to fuse a gene of the
desired photosensitizer protein with a known CPP, here PEN taken as an example, the
30 KR gene was cloned into a vector that already had the PEN-CPP gene cloned into it

(pETDuet-1-PEN vector). PEN gene was cloned in this vector between BamHI and HindIII sites. To extract the KR gene from pET28c vector, it was first amplified using “T7 promoter forward primer” having polynucleotide sequence as set forth in SEQ ID NO. 18 and a “KR specific reverse primer – 1” having polynucleotide sequence as set forth in SEQ ID NO. 19). The amplified KR gene was then digested with NcoI and NheI restriction sites and cloned into the pETDuet-1 vector with PEN-CPP. The positive clone was then further digested with BamHI site and self-ligated. The ligated product was transformed into chemical competent Top10 cells purchased from Thermo Fisher Scientific (cat. C404010). To check for positive clones, the clones were screened with “Duet 5’ forward primer” having polynucleotide sequence as set forth in SEQ ID NO. 20) and “KR specific reverse primer-1”. Initially this construct was unstable and degraded during gel filtration. Therefore, based on computational analysis the C-terminal region prone to degradation was truncated to yield fusion proteins with improved stability and cell penetrating properties. The truncated version was prepared by amplifying the complete pETDuet-1 KR-PEN vector with a single primer, “KR specific reverse primer – 2” having polynucleotide sequence as set forth in SEQ ID NO. 21. This amplification incorporated BamHI restriction site. Further, digestion with BamHI restriction enzyme followed by ligation removed the C-terminal 11 amino acid residues (RITSAIGSDEDGS; amino acid sequence as set forth in SEQ ID NO. 22). The ligated product was then transformed to chemical competent Top10 cells and the positively screened colonies were cultured for plasmid isolation. A similar construct was designed for cloning and expression of a homologous GFP gene with the same CPP-PEN. This construct was used to generate GFP-PEN fused protein that would internalize similar to this invention. GFP is also a known photosensitizer, but does not produce ROS as efficiently as KR, and so does not lead to cell killing. Keeping this fact, we used the GFP-PEN fusion protein as a control in our experiments.

Example 2

Purification of fusion protein

All positive clones were sequenced and transformed in chemical competent BL21 (DE3) to express the desired fusion protein. The growing cultures were induced at 0.6 OD with

0.3 mM IPTG and incubated at 18°C overnight with continuous shaking at 200 rpm in an incubator shaker. The cells were harvested at 9000xg for 20 min and resuspended in 1X Phosphate Buffer Saline (1X PBS buffer-137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4) buffer. The red color of the pellet is a positive indication of the over expression of KR proteins. The cells were further lysed by sonication, and the soluble colored fractions were obtained by centrifuging the lysates at high-speed 18000xg for 60 min. The insoluble pellet fractions were discarded, and the colored supernatants were then mixed with Ni-NTA resin for binding. The proteins were eluted using the different gradient of imidazole, preparing in 1XPBS buffer pH 7.4. SDS-PAGE (15%) was run according to the protocol developed by Laemmli (*Laemmli 1970*) (Figure 2Band 2D). Approximately 10µg of protein samples were mixed with 5X sample buffer (0.25M Tris-HCl, pH 6.8. 15% SDS. 50% glycerol. 25% β-mercaptoethanol. 0.01% bromophenol blue). The protein samples were denatured by boiling and centrifuged at 12,000 rpm for 5 minutes each. The discontinuous gel system was used having different concentration and pH of the resolving and stacking components. Resolving gel was casted in 1.5M Tris-Cl, pH-8.8 and different poly-acrylamide concentrations depending on the size of the proteins. Purified proteins were further concentrated using Amicon® Ultra-15 10K Centrifugal Filter Devices (Merk USA). Gel filtration of the fusion proteins were done using the BIO-RAD NGC™ Chromatography system with Superdex™ 200; 10/300 GL column at a flow rate of 0.5 ml/min. The fractions falling under the peak of elution were pooled and concentrated upto 1 ml volume (Figure 2C). This concentrated protein was filter sterilized using a 2µm filter and sealed for use in the cell culture experiments.

Example 3

Monitoring internalization of the fusion protein (KR-PEN) in cancer cells mediated by CPPs

The HeLa cell line was procured from ATCC (American Type Culture Collection, USA). The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) in high glucose media procured from Sigma. 10% fetal bovine serum (FBS) was added for maintaining the cell line. The cells were passaged every alternate day on observing confluence. For the internalization experiments, the Hela cells when adhered to and

confluent, were washed off to remove complete media and incubated with 10 μ M concentration of the KR-PEN in fasted DMEM (without FBS) in a 6 or 12 well plate at a temperature of 37 °C. Post a timed incubation of 2, 4, 8 and 12 hours, the cells were washed with 1X PBS and trypsinized with Trypsin-EDTA (Ethylenediamine Tetraacetic Acid, buffered solution) from Sigma. Trypsinized cells were harvested after centrifugation at different time intervals of 2, 4, 8 and 12 hours and studied for protein internalization using the FACS Aria flow cytometer from BD Biosciences. The cells were acquired via the PE-Texas Red laser and the FITC laser to cover for both red for KR and green for GFP fluorescence wavelengths. Figure 4 shows the results of cell internalization experiments, FACS & Confocal in HeLa cells. The novel fusion protein internalized the cells to more than 90% in 4 hours.

Example 4

Killing of the targeted cancer cells by exposure to light source

Now, to check the photodynamic efficiency of the internalized protein, the cells were seeded with 10 μ M concentration of the fusion KR protein for 4 hours at a temperature of 37 °C. These were then stained with working concentration of 5 μ g/ μ l of Hoechst 33342 stain procured from Sigma, USA. Post nuclei staining with Hoechst 33342, the cells were washed and replaced with fresh complete DMEM with 10% FBS and exposed to a green light source of 1Watt again for predetermined time points of 10, 20, 30 and 45 minutes. The cells at each time point were washed with 1X PBS and the cover slips mounted upside down on the slides for viewing under the confocal microscope.

The images in phase contrast, DAPI (4', 6-diamidino-2-phenylindole) and Texas red were taken for viewing whole cells, nuclei and the KR, respectively using the Olympus 1X80 confocal microscope. Figure 5 shows the photodynamic killing of the cells internalizing the photosensitizer protein, time as a variable factor. It was observed that KR internalized efficiently inside the HeLa cells and after 30 minutes of light source exposure, was able to kill the cells in which it internalized. The cells that were not exposed to any light source, no significant cell killing was observed confirming that the light source alone did not contribute to any killing and that only the photosensitizer effect of the KR caused the cells to die. Also, the cells that internalized GFP-PEN were not killed despite exposure

to the light source, suggesting CPP fusion to be an inefficient photosensitizer is not cytotoxic.

The present invention provides a strategy of fusing a gene of a photosensitizer with the gene of a CPP, to create a fusion protein, which is capable of internalizing inside the cells which are targets of the CPPs used. The photosensitizer protein expresses well and is functional despite fused to the CPPs and is able to produce ROS of the desired level so as to be able to kill the cells where it is internalized.

ADVANTAGES OF THE INVENTION

1. The key advantage of the present invention is that it is able to bring out a construct of an efficient photosensitizer, KR, with a CPP, that can be expressed and made available for tumor internalization and killing, upon green light source irradiation, from an affordable *E. coli* expression system.
2. This entire process of expressing the fusion protein of a photosensitizer along with a CPP, in a bacterial system makes this anti-cancer treatment strategy an affordable one that can help provide an efficient and targeted therapy to all.
3. The concept of fusion protein generation as disclosed in the present invention helps save a lot of resources and time, as in expressing and purifying the two essential components separately, and then covalently linking them to bring out a stable product that performs the functions as efficiently as expected of the individual components.
4. The fusion protein of the present invention is soluble and stable and demonstrates efficient cancer cell internalization for its specific cell killing effect to happen.

Table 1: Fusion Proteins of the present invention

S. No.	Fusion protein Name	SEQ ID No.	Specific cancer type
1.	KR	SEQ ID NO. 1	Not applicable
2.	KR-PEN	SEQ ID NO. 2	HeLa and Hep3B Cells
3.	GFP-PEN	SEQ ID NO. 3	HeLa and Hep3B Cells
4.	KR-MAP	SEQ ID NO. 4	HeLa and Hep3B Cells
5.	KR-Pep5	SEQ ID NO. 5	HeLa cells
6.	KR-Glu-oct-6	SEQ ID NO. 6	Prostate cancer cells
7.	KR-B1	SEQ ID NO. 7	MCF-7 CELLS
8.	KR-Ds4.3	SEQ ID NO. 8	HeLa and Hep3B Cells
9.	KR-Peptide1-NsD	SEQ ID NO. 9	Glioblastoma
10.	KR-YTA2	SEQ ID NO. 10	Breast cancer
11.	KR-CPP1	SEQ ID NO. 11	Hepatocellular carcinoma cell line, Hepatocellular carcinoma cell line, Normal hepatocyte line
12.	KR-CPP2	SEQ ID NO. 12	HUVEC, HMVECd, A375M, MCF-7, MDA-MB-231, HeLa, HT-29, Jurkat, CEM and fresh peripheral blood mononuclear cells
13.	KR-CPP3	SEQ ID NO. 13	MCF-7, DU145, A375 HeLa, A875, M14, A549, ZR-75-30, Raji, NB4, K562 cell lines, lung cancer, gastric cancer, neuroblastoma
14.	KR-CPP4	SEQ ID NO. 14	Prostate, Pancreatic ductal, breast cancer
15.	KR-CPP5	SEQ ID NO. 15	Lymphatic cancer

16.	KR-TAT	SEQ ID NO. 16	Prostate, Pancreatic ductal, breast cancer, lung cancer
17.	KR-Br2	SEQ ID NO. 17	Breast cancer

Table 2: List of primers (SEQ ID 18 to 21)

Sl No.	Primer Name	Sequence
1.	T7 Promoter	5'-taatacgactcactataggggaattg-3' (SEQ ID NO. 18)
2.	KR specific reverse primer - 1	5'-agctgctagcggatccgtcttcacggagccgatcg-3' (SEQ ID NO. 19)
3.	Duet 5' forward	5'ctgaaagacgcgcagactaattcg-3' (SEQ ID NO. 20)
4.	KR specific reverse primer - 2	5'-gagccgatcgactcgtggatcccgaacagatgggcgtac-3' (SEQ ID NO. 21)

Table 3: List of plasmid DNA construct

S.N.	Plasmid DNA construct Name	SEQ ID NO
1.	pET28c-KR	SEQ ID NO. 23
2.	pETDuet-1 KR-PEN	SEQ ID NO. 24
3.	pETDuet-1 GFP-PEN	SEQ ID NO. 25
4.	pETDuet-1 KR-MAP	SEQ ID NO.26
5.	pETDuet-1 KR-Pep5	SEQ ID NO.27
6.	pETDuet-1 KR-Glu-oct-6	SEQ ID NO.28
7.	pETDuet-1 KR-B1	SEQ ID NO.29
8.	pETDuet-1 KR-Ds4.3	SEQ ID NO.30
9.	pETDuet-1 KR-Peptide1-NsD	SEQ ID NO.31
10.	pETDuet-1 KR-YTA2	SEQ ID NO.32
11.	pETDuet-1 KR-CPP1	SEQ ID NO.33
12.	pETDuet-1 KR-CPP2	SEQ ID NO.34
13.	pETDuet-1 KR-CPP3	SEQ ID NO.35
14.	pETDuet-1 KR-CPP4	SEQ ID NO.36
15.	pETDuet-1 KR-CPP5	SEQ ID NO.37
16.	pETDuet-1 KR-TAT	SEQ ID NO.38
17.	pETDuet-1 KR-Br2	SEQ ID NO.39

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Foreign patents

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We Claim:

1. A fusion protein for cancer therapy consisting of:
 - a. a photosensitizer protein; and
 - b. a cell penetrating peptide.
- 5 2. The fusion protein as claimed in claim 1 wherein the photosensitizer protein is KR.
3. The fusion protein as claimed in claim 1 wherein the fusion protein is a protein having amino acid sequence selected from the group consisting of SEQ ID NOs. 2 and 4-17.
- 10 4. A plasmid DNA construct comprising a gene encoding the fusion protein as claimed in claim 1 having the polynucleotide sequence selected from the group consisting of SEQ ID NO. 24 and 26 to 39.
5. The plasmid DNA construct as claimed in claim 4 wherein said construct is expressed in *Escherichia coli* cells.
- 15 6. A method for targeting the fusion protein as claimed in claim 1 into a cell comprising the steps of:
 - a. incubating the fusion protein as claimed in claim 1 with cells at 37 °C for 30 minutes to 12 hours; and
 - b. activating the fusion protein by exposure to a visible light, preferably
 - 20 green light.
7. The method as claimed in claim 6, wherein the cell is selected from the group consisting of HeLa cell, Hep3B cell, prostate cancer cell, MCF-7 cell, glioblastoma cell, breast cancer cell, hepatocellular carcinoma cell, normal hepatocyte cell, HUVEC cell, HMVECd cell, A375M cell, MCF-7 cell, 25 MDA-MB-231 cell, HT-29 cell, Jurkat cell, CEM cell, peripheral blood mononuclear cell, DU145 cell, A875 cell, M14 cell, A549 cell, ZR-75-30 cell, Raji cell, NB4, K562 cell, lung cancer cell, gastric cancer cell, neuroblastoma cell, pancreatic ductal cancer cell, lymphatic cancer cell, and lung cancer cell.

8. The fusion protein as claimed in claim 1 for use in attaching to upconversion nanoparticles.

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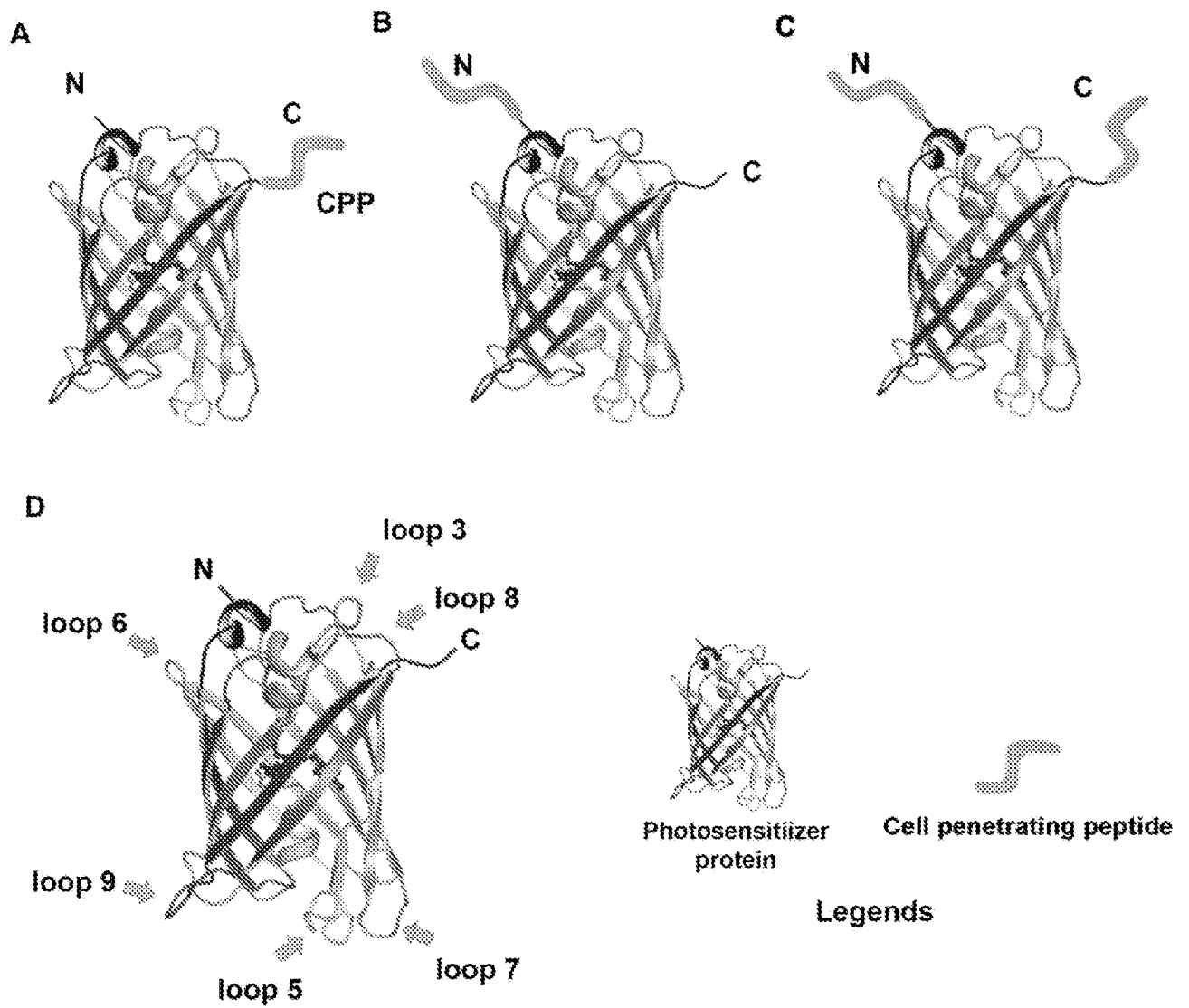
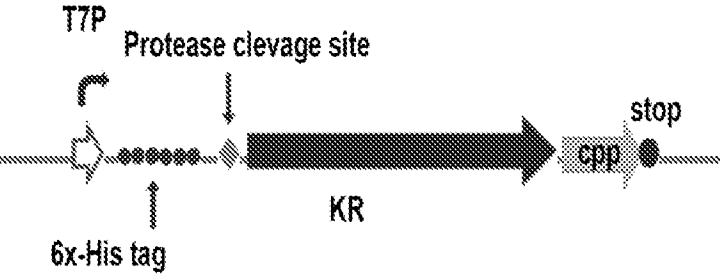
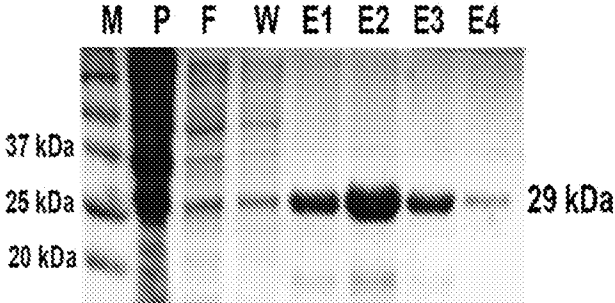


FIG. 1

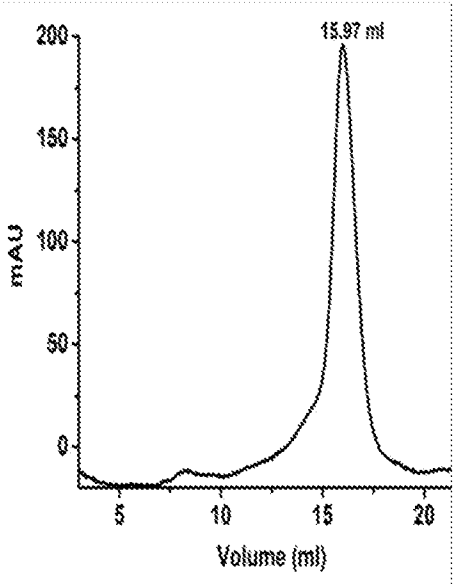
A



B



C



D

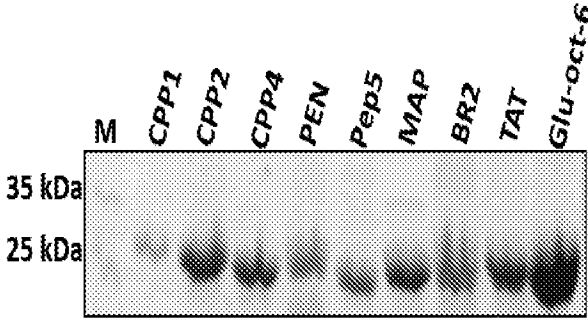


FIG. 2

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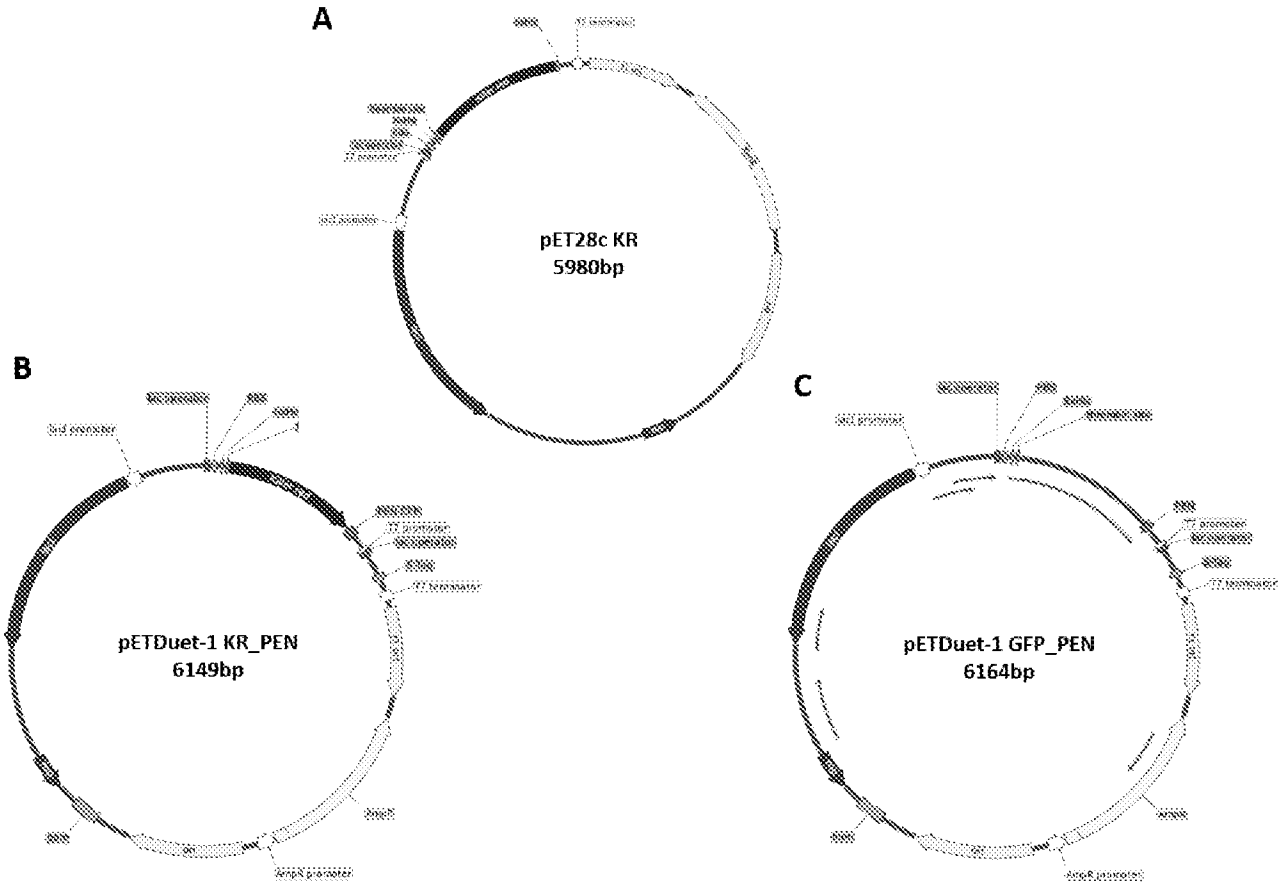


FIG. 3

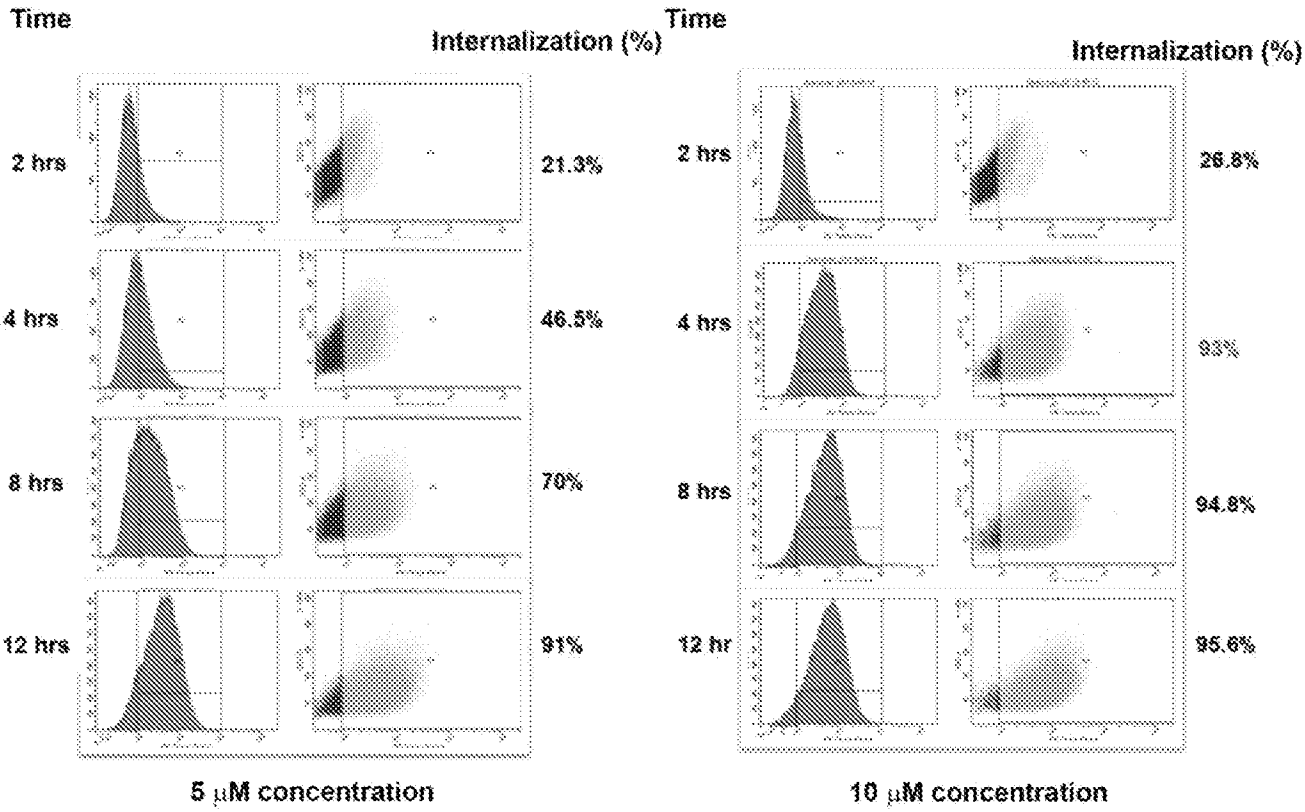


FIG. 4

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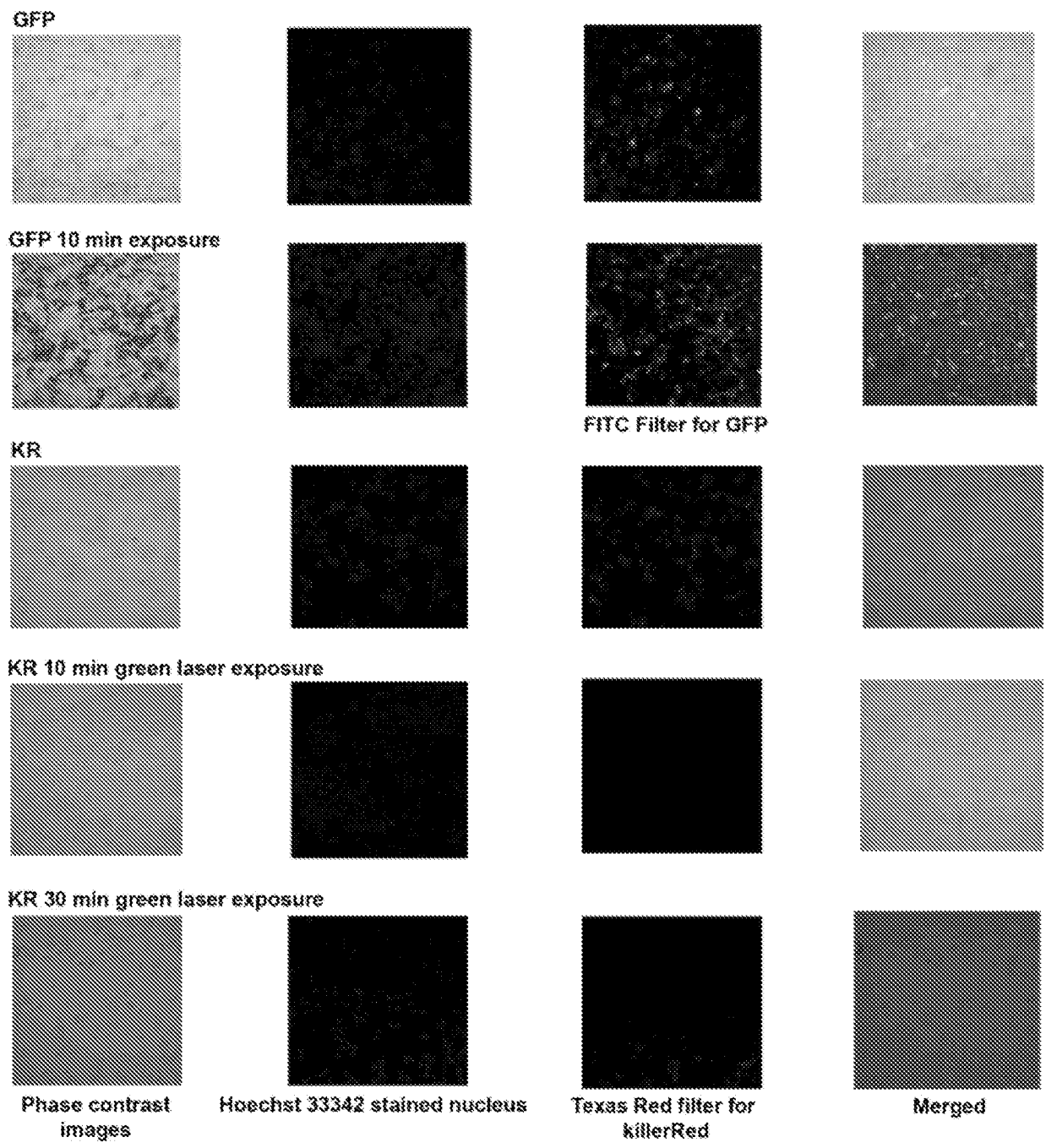


FIG. 5

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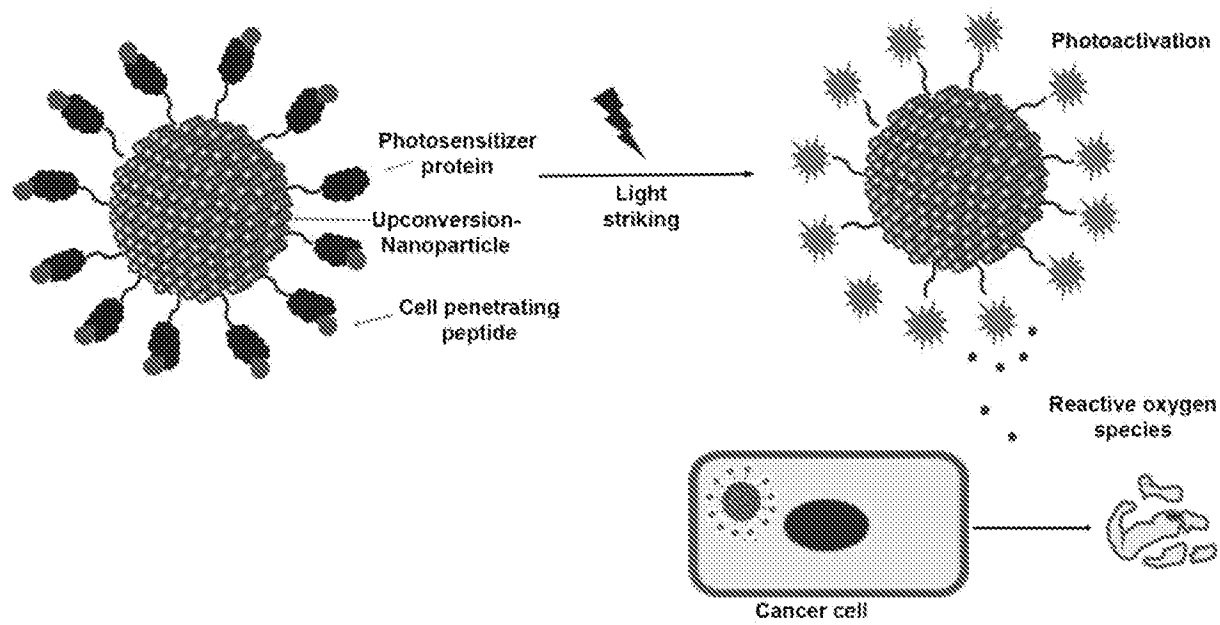


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/050272

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/00,A61K47/64 Version=2020.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN107141354 (A) (LI SIWEN; ZHAO LEI) 08-09-2017 (08 September, 2017) Abstract	1-5, 8
Y	Choi Y, McCarthy JR, Weissleder R, Tung CH. "Conjugation of a photosensitizer to an oligoarginine-based cell-penetrating peptide increases the efficacy of photodynamic therapy". ChemMedChem: Chemistry Enabling Drug Discovery. 2006 Apr 10;1(4):458-63. Abstract, page 458	1-5, 8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23-07-2020

Date of mailing of the international search report

23-07-2020

Name and mailing address of the ISA/

Indian Patent Office
Plot No.32, Sector 14, Dwarka, New Delhi-110075
Facsimile No.

Authorized officer

P Jyothish Kumar

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/050272

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 6 and 7
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 6, 7 relates to a method for treatment, which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.