TREATMENT OF CIRCULATING TUMOR CELLS USING AN EXTRACORPOREAL DEVICE

Applicant: LI-COR, INC., Lincoln, NE (US)

Inventors: JOY L. KOVAR, Lincoln, NE (US); HAN-WEI WANG, Lincoln, NE (US)

Assignee: LI-COR, INC., Lincoln, NE (US)

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Related U.S. Application Data


ABSTRACT

The present invention relates to methods and compositions for eliminating circulating tumor cells (CTCs) in a patient with or without solid tumors. In particular, the ex vivo method utilizes a photodynamic or magnetic targeting moiety that specifically binds to antigens expressed on the cell surface of CTCs.

Diagram:

- 100. Extracorporeal Circuit
- 110. PDT agent conjugation section
- 120. Blood line, inlet
- 121. Blood line, reintroducing
- 130. Radiation chamber and agent administration
- 140. Radiation controller
- 150. Blood line, inlet
- 151. Blood line, reintroducing

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<table>
<thead>
<tr>
<th>FIG. 3A</th>
<th>FIG. 3B</th>
<th>FIG. 3C</th>
<th>FIG. 3D</th>
<th>FIG. 3E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase</strong></td>
<td><strong>700nm</strong></td>
<td><strong>Composite</strong></td>
<td><strong>Phase</strong></td>
<td><strong>700nm</strong></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
<td>EGF-700DX (1.5 µM)</td>
<td>EGF-700DX (1.5 µM)</td>
</tr>
<tr>
<td>No EGF-700DX</td>
<td>No EGF-700DX</td>
<td>EGF-700DX (1.5 µM)</td>
<td>IR (8 J/cm²)</td>
<td>IR (16 J/cm²)</td>
</tr>
<tr>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**EGF-700DX** (1.5 µM) IR (8 J/cm²)
FIG. 8
FIG. 9A

Box-Cox Plot for Power Transforms

FIG. 9B

Normal Plot of Residuals

Externally Studentized Residuals
**FIG. 9C**

ANOVA for selected factorial model

Analysis of variance table (Partial sum of squares - Type III)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Prob &gt; F</th>
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<tbody>
<tr>
<td>Model</td>
<td>92.56</td>
<td>7</td>
<td>13.22</td>
<td>18.16</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A-Concentration</td>
<td>0.38</td>
<td>1</td>
<td>0.38</td>
<td>0.29</td>
<td>0.5988</td>
</tr>
<tr>
<td>B-Irradiation</td>
<td>85.88</td>
<td>1</td>
<td>85.88</td>
<td>66.00</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C-Incubation</td>
<td>2.04</td>
<td>1</td>
<td>2.04</td>
<td>1.57</td>
<td>0.2284</td>
</tr>
<tr>
<td>A*B</td>
<td>1.60</td>
<td>1</td>
<td>1.60</td>
<td>1.23</td>
<td>0.2836</td>
</tr>
<tr>
<td>A*C</td>
<td>0.042</td>
<td>1</td>
<td>0.042</td>
<td>0.032</td>
<td>0.8802</td>
</tr>
<tr>
<td>B*C</td>
<td>2.53</td>
<td>1</td>
<td>2.53</td>
<td>1.95</td>
<td>0.1819</td>
</tr>
<tr>
<td>A<em>B</em>C</td>
<td>0.082</td>
<td>1</td>
<td>0.082</td>
<td>0.063</td>
<td>0.8054</td>
</tr>
<tr>
<td>Pure Error</td>
<td>20.82</td>
<td>16</td>
<td>1.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>113.38</td>
<td>23</td>
<td></td>
<td></td>
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</tbody>
</table>
**FIG. 10**

![Bar chart showing vitality over time for different conditions.](chart.png)
TREATMENT OF CIRCULATING TUMOR CELLS USING AN EXTRACORPOREAL DEVICE

CROSS-REFERENCE TO RELATED APPLICATIONS


BACKGROUND OF THE INVENTION

[0002] Tumor metastasis is the process in which cancer cells spread from the site of a primary solid tumor to one or more other organs in the body. The cancer cells, such as circulating tumor cells (CTCs), shed from the solid tumor, translocate to distant tissues through blood and lymphatic vessels, and eventually proliferate and colonize to form metastases.

[0003] Solid tumors are treated by therapies including surgery, radiotherapy, chemotherapy and targeted antibody therapy. Photodynamic therapy using photosensitizing moiety or biomolecule-fluorophore conjugates has been widely described as a possible treatment as well (see, e.g., U.S. Pat. No. 8,524,239).

[0004] Photosensitizing antibody–fluorophore conjugates can utilize phthalocyanine dyes, such as IRDye® 700DX (LI-COR Biosciences, Lincoln, Nebr.) which has low sensitivity to photobleaching and is highly photostable (see, e.g., U.S. Pat. No. 7,005,518). These dyes can be conjugated to various biomolecules, such as antibodies or fragments thereof, antibodies, nanobodies, Affibodies®, diabodies, minibodies, antigens, ligands, proteins, peptides, carbohydrates, nucleic acids, small molecules, pseudovirion, etc. When exposed to excitation light, the phthalocyanine dye generates a cytotoxic singlet oxygen that can induce cell death, such as through apoptosis, necrosis or autophagy. Two types of reactions occur, Type I generates radicals and Type II generates singlet oxygen. Both radicals and singlet oxygen cause oxidative destruction of tissues. It has been shown that the administration of photodynamic therapy using IRDye® 700DX-anti-HER2™ antibodies can decrease the size of HER2™ solid tumors in a mouse model (Mitsunaga et al., Nat. Med., 2011, 17(12):1685-1691).

[0005] Photodynamic therapy with photosensitizer can be performed with different forms of light sources (see, e.g., U.S. Pat. Nos. 5,976,175 and 8,175,687) to enable excitation light reaching different body locations. Whole body exposure with tanning bed (U.S. Pat. No. 6,896,693) or long-term treatment with wearable devices (U.S. Pat. No. 7,850,720) has been demonstrated. One of key factors in these kinds of methods is the extensive extinction of excitation light when passing through skins, soft tissues, or organs.

[0006] Extracorporeal blood treatment eliminates the concern of light absorption through thick tissue layers. Currently, extracorporeal devices have been applied to patients for different treatment such as hemodialysis, extracorporeal oxygenation, CO₂ removal, and apheresis, etc. The knowledge on extracorporeal circulation is thus well established and broadly applied. With the concept of extracorporeal light treatment on blood components, photopheresis utilizes separation of blood compositions and applies UV light to the concentrated part (e.g. white blood cells) before returning the recombined blood components to the patient. The photopheresis or extracorporeal photochemotherapy (see, e.g., U.S. Patent Publication Nos. 2003/0139466 and 2013/0197419) is an immunomodulating procedure that has been available for the treatment of cutaneous T-cell lymphoma such as mycosis fungoides or Sézary syndrome. Photopheresis is also used to treat conditions including graft-versus-host disease and organ transplant rejection. However, the rare CTCs shed from solid tumor into blood stream could not be separated and concentrated from blood effectively in the online extracorporeal device. The invention provides methods for extracorporeal treatment of CTCs.

[0007] Clinically, the number of CTCs in a patient can be estimated via the CELSEARCH® System (Janssen Diagnostics, LLC, Raritan, N.J.). The system utilizes ferrofluid nanoparticles with epithelial cell adhesion targeting antibodies to enumerate CTCs from the bulk of 7.5 ml blood samples. The CTCs are then distinguished with immunofluorescence dyes under a microscopic imaging system for diagnostic purpose. The system renders the monitoring of metastatic cancer patients throughout treatment to making informed clinical decisions.

[0008] Current treatment methods for solid tumors are not effective at eliminating CTCs since they target only the cells of the tumor. There is a need for an effective method of reducing the number of CTC, and thereby reduce the risk of metastasis. The present invention satisfies this need and provides related advantages as well.

BRIEF SUMMARY OF THE INVENTION

[0009] In one aspect of the present invention, provided herein is a method for inducing apoptosis, necrosis or autophagy resulting in cell death of circulating tumor cells (CTCs) in a subject. The method comprises (a) administering to the subject’s blood a therapeutically effective agent comprising a near-infrared phthalocyanine dye conjugated to an moiety or biomolecule that specifically binds to CTCs; (b) ex vivo irradiating the subject’s blood with an appropriate excitation light in an amount to effectively induce apoptosis, necrosis and/or autophagy in the CTCs; and (c) re-introducing the treated blood into the subject.

[0010] In some embodiments, the subject has a solid tumor or has had a solid tumor. In some instances, the solid tumor is a breast tumor, colorectal tumor, lung tumor, prostate tumor, ovarian tumor, pancreatic tumor, liver tumor, or bladder tumor.

[0011] In some embodiments, the phthalocyanine dye is IRDye™ 700DX. In some embodiments, the light has a wavelength of about 660 to 740 nm.

[0012] In some embodiments, the moiety or biomolecule specifically binds a CTC antigen. In some instances, the CTC antigen is selected from the group consisting of epithelial cell adhesion molecule (EpCAM), cytokeratin 8, cytokeratin 18, cytokeratin 19, E-cadherin, fibronectin, vimentin, MUC1, Her2, Twist, E-selectin, HIP8G, CD44, octreot, and others.

[0013] In some embodiments, the therapeutically effective agent is administered at the time of anticoagulant administration with heparin, sodium citrate, thrombin inhibitors, protacelycin and other prostaglandin or other effective anticoagulants.

[0014] In some embodiments, ex vivo irradiating is performed using an extracorporeal device.

[0015] In some embodiments, un-bound therapeutically effective agent will be reduced or removed from blood via filtration or dialyzer or methods that involved molecular movement.
In another aspect of the present invention, provided herein is a method of extracorporeal treatment of cancer in a subject having circulating tumor cells (CTCs). The method comprises: (a) intravenously administering a therapeutically effective agent comprising a phthalocyanine dye conjugated to a targeting moiety that specifically binds to CTCs; (b) ex vivo irradiating the subject's blood with a light (e.g., wavelength of 660 to 740 nm) in an amount to effectively induce apoptosis, necrosis or autophagy in CTCs; and (c) re-introducing the irradiated blood into the subject, thereby treating cancer in the subject.

In some embodiments, step (b) and/or (c) are performed using an extracorporeal device.

In some embodiments, the subject has undergone solid tumor resection.

In some embodiments, the therapeutic agent is IRDye® 700DX. In some embodiments, the light has a wavelength of 660 to 740 nm.

In some embodiments, the antibody specifically binds a CTC antigen. In some instances, the CTC antigen is selected from the group consisting of epithelial cell adhesion molecule (EpCAM), cytokeratin 8, cytokeratin 18, cytokeratin 19, E-cadherin, fibronectin, vimentin, MUC1, Her2, Twist, E-selectin, HSPG, CD44, octreotide, and others.

In some embodiments, the therapeutically effective agent is administered at the time of anticoagulant administration with heparin, sodium citrate, thrombin inhibitors, protacyclin and other prostanoiads or other effective anticoagulants.

In some embodiments, unbound therapeutically effective agent will be reduced or removed from blood via filtration or dialyzer or methods that involved molecular movement.

In some embodiments, ex vivo irradiating is performed using an extracorporeal device.

In another aspect of the present invention, provided herein is a method of extracorporeal treatment of cancer in a subject having CTCs. The method comprises: (a) administering a targeting agent comprising a magnetic particle conjugated to a biomolecule that specifically binds to CTCs; (b) ex vivo treatment of CTCs with an extracorporeal circuit to separate the particle-bound CTCs from blood stream with magnetic influence(s); and (c) re-introducing the treated blood into the subject, thereby mitigate the number of CTCs in the subject.

In some embodiments, the treatment agent is introduced ex vivo through the extracorporeal device.

In some embodiments, step (b) and/or (c) are performed using an extracorporeal device.

In some embodiments, the subject has undergone solid tumor resection.

In some embodiments, the antibody specifically binds a CTC antigen. In some instances, the CTC antigen is selected from the group consisting of epithelial cell adhesion molecule (EpCAM), cytokeratin 8, cytokeratin 18, cytokeratin 19, E-cadherin, fibronectin, vimentin, MUC1, Her2, Twist, E-selectin, HSPG, CD44, octreotide, and others.

In some embodiments, the magnetic particle-targeted CTCs is removed or separated from blood via methods that involve molecular movement such as the influence of magnetic field(s).

In yet another aspect of the present invention, provided herein is a therapeutically effective composition comprising a phthalocyanine dye conjugated to a circulating tumor cell (CTC) targeting moiety or biomolecule that specifically binds to a CTC.

In some embodiments, the phthalocyanine dye is IRDye® 700DX.

In some embodiments, the targeting moiety or biomolecule specifically binds a CTC antigen. In some instances, the CTC antigen is selected from the group consisting of epithelial cell adhesion molecule (EpCAM), cytokeratin 8, cytokeratin 18, cytokeratin 19, E-cadherin, fibronectin, vimentin, MUC1, Her2, Twist, E-selectin, HSPG, CD44, octreotide, and others.

Other objects, features and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of an exemplary embodiment in which the CTC extracorporeal photodynamic treatment is performed in the extracorporeal circuit.

FIG. 2 illustrates an exemplary embodiment of the methods with CTC targeting magnetic particles which attaches to CTCs and renders the removal of CTCs with magnetic influence (e.g., a magnetic field).

FIGS. 3A-E show a reduction in the number of targeted cancer cells after exposure to irradiating light at 8 J/cm² or 16 J/cm². FIGS. 3A-C show control data and FIGS. 3D-E show exposure to 8 J/cm² or 16 J/cm², respectively.

FIGS. 4A-D show a reduction in the number of targeted cancer cells after exposure to irradiating light at 670 nm to 700 nm at 8 J/cm² or 16 J/cm² was made through 1 mm whole blood. FIGS. 4A-B present control data and FIGS. 4C-D show exposure to 8 J/cm² or 16 J/cm², respectively.

FIGS. 5A-B show the IRDye® 700DX-EGF probe binding to A431 cells at two concentrations for two different time periods. A431 cells received a peristaltic flow of 1.36 ml/min of IRDye® 700DX-EGF at a concentration of 0.2 μM for 15 min (FIG. 5A). A431 cells received a peristaltic flow of 1.36 ml/min of IRDye® 700DX-EGF at a concentration of 0.5 μM for 5 min (FIG. 5B).

FIGS. 6A-6D show images of A431 cells incubated for 5 minutes with variable concentrations of IRDye 700DX-EGF and then irradiated at 4 J/cm². FIG. 6A shows the cells during the incubation of 0.5 μM of probe and FIG. 6B shows the cells after irradiation. FIG. 6C shows the cells incubated with 2 μM of probe. FIG. 6D shows these cells after irradiation.

FIGS. 7A-7D provides images of A431 cells incubated for 10 minutes with variable concentrations of IRDye 700DX-EGF and then irradiated at 16 J/cm². Cells that were incubated with 0.5 μM of probe are shown in FIG. 7A. FIG. 7B shows these cells after irradiation. FIG. 7C shows cells treated with 2 μM of probe and FIG. 7D shows the cells after irradiation treatment.

FIG. 8 illustrates that the percent cell vitality was lower for irradiation treatments of 16 J/cm² (e.g., treatments 3, 4, 7 and 8) compared to those of 4 J/cm². Within the parameters tested, cell vitality was independent of probe concentration and probe incubation times.

FIGS. 9A-C provides data of the statistical analysis performed. FIG. 9A shows the Box-Cox plot indicating that no transformation of data was required. FIG. 9B shows the normal probability plot of residuals that indicates that the
residuals followed a normal distribution and were a good fit. FIG. 9C shows the ANOVA analysis.

[0043] FIG. 10 provides a graph of the percent cell vitality of tumor cells treated with IRDye® 700DX-EGF or IRDye® 700DX-panitumumab and irradiated at 24 J/cm². The cells were incubated for 5 minutes, 10 minutes or 20 minutes with the probe.

[0044] FIGS. 11A-C provides images of the cells after irradiation at 24 J/cm². The cells exhibited morphological blebbing which is associated with apoptosis and/or necrosis (see, white arrows).

DETAILS DESCRIPTION OF THE INVENTION

I. Introduction

[0045] The present invention relates to compositions and methods for the elimination of CTCs circulating in the blood of an individual. More particularly, the invention involves the use of a continuous-flow, extracorporeal device that treats the cells with IRDye®-targeting agents or magnetic particle-targeting agent to either trigger CTC cell death or enable the removal of CTCs from the blood stream before returning the treated blood to the body. Cell death of CTCs can be induced with an IRDye®/700DX-targeting agent and the appropriate light exposure. In another aspect of the present invention, the CTCs can be removed from the blood using a magnetic particle-targeting agent and a magnetic influence.

II. Definitions

[0046] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0047] The terms “a,” “an,” or “the” as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a cell” includes a plurality of such cells and reference to “the agent” includes reference to one or more agents known to those skilled in the art, and so forth.

[0048] The term “CTC targeting moiety” refers to any compound, molecule, or fragment thereof that can specifically bind to the surface of a circulating tumor cell (CTC), e.g., any component present on the surface of a CTC, and/or tumor cell matter, e.g., any matter or component thereof that is derived, originated, or secreted from a tumor cell. Molecules that can be used as CTC targeting moieties include, but are not limited to, peptides, polypeptides, proteins, peptidomimetics, ligands, antigens, antibodies, antibody fragments (e.g., antigen binding fragments of antibodies), nanobodies, Affibodies®, diabodies, minibodies, carbohydrate-binding proteins, e.g., a lectin, glycoproteins, glycoprotein-binding molecules, amino acids, carbohydrates (including mono-, di-, tri- and poly-saccharides), lipids, steroids, hormones, lipid-binding molecules, cofactors, nucleosides, nucleotides, and nucleic acids (e.g., DNA or RNA, analogues and derivatives of nucleic acids, or aptamers), peptidoglycans, lipopolysaccharides, small molecules, and any combinations thereof.

[0049] The term “antibody” refers to a polypeptide comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen, such as a tumor-specific protein. Antibodies are composed of a heavy and a light chain, each of which has a variable region, termed the variable heavy (VH) region and the variable light (VL) region. Together, the VH region and the VL region are responsible for binding the antigen recognized by the antibody. Antibodies include intact immunoglobulins and the variants and portions of antibodies well known in the art, such as Fab fragments, Fab' fragments, F(ab')2 fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, Ill.); Kuby, J., Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997.

[0050] The term “IRDye® 700DX” or “700DX” refers to a dye having the preferred NHS ester linkage to allow for conjugation to a CTC targeting moiety. Typically, the CTC targeting moiety such as an antibody has a primary amine (e.g., an amino group) wherein the NHS ester and the amino group react to form an amide bond, linking the targeting moiety such as an antibody to 700DX. The NHS ester IRDye® 700DX has the following formula:
[0051] The dye is commercially available from LI-COR (Lincoln, Nebr.). Amino-reactive IRDye® 700DX is a relatively hydrophilic dye and can be covalently conjugated with an antibody using the NHS ester of IRDye® 700DX. Other variations of IRDye 700DX are disclosed in U.S. Pat. No. 7,005,518 (incorporated herein by reference), and those too are useful in the present invention. The carbamate derivative has the following name and structure, silica(5), bis[N3-[(hydroxy-kappa-O)-dimethylsilylpropyl]-3-sulfo-NN-bis (3-sulfopropyl)-1-propanaminiumato(4+)]iN3-[(2H, 31H)-phthalocyaninato-kappa.N32)kappa.N30, kappa.N31, kappa.N32]oxy[propoxy]carbonylaminohexameto(3+)]- sodium (1:5); CAS Registry Number: [1623074-46-3];

[0052] The term “photodynamic therapy,” “PDT” or “photoinmunotherapy” refers to a molecular targeted therapy that utilizes a target-specific photosensitizer agent, based on a near infrared (NIR) phthalocyanine dye, IRDye® 700DX, conjugated to a targeting moiety that specifically bind to a cell surface protein. For example, the cell surface protein such as EpCam is found specifically on circulating tumor cells, and thus photodynamic therapy can be used to kill such cells. Cell death of the cells can be induced when the IRDye® 700DX anti-EpCam antibody binds to the cells and the cells are irradiated with the proper excitation light. For most application of PDT, the cytotoxic agent is produced by one of the two different processes called Type I or Type II PDT pathways. Type I generates radicals and Type II generates singlet oxygen. Both radicals and singlet oxygen cause oxidative destruction of tissues.

[0053] Non-targeted options for PDT include sensitizers, porphyrins, chlorophylls and dyes.

[0054] Non-limiting examples of useful photosensitizers include aminolevulinic acid (ALA), silicon phthalocyanine Pe 4, m-tetrahydroxyphenylchlorin (mTHPC), mono-L-aspartyl chloride 6 (NPe6), and methylene blue. Commericially photosensitizers available for clinical use include Alhumera®, Photofrin®, Visudyne®, Levulan®, Foscan®, Metvix®, Hexvix®, Cysview®, and Laserphryin®. Other photosensitizers in development include Antrin®, Photocrom®, Photosens, Photorex®, Lumacen®, Cevira®, Visona®, BF-200 AL-A®, Amphelinos®, and Azacypromethene®. Other classes of PDT agents include halogenated compounds, such as those described in, e.g., U.S. Patent amount of the therapeutic composition (such as an IRDye® 700DX-CTC targeting moiety) can be dependent on several factors, including, but not limited to the subject or cells being treated, the particular therapeutic agent, and the manner of administration of the therapeutic composition. For example, a therapeutically effective amount or concentration is sufficient to prevent advancement (such as metastasis), delay progression, or to cause regression of a disease, or which is capable of reducing symptoms caused by the disease, such as cancer. For instance, a therapeutically effective amount or concentration is sufficient to increase the survival time of a patient with a circulating tumor cell.

[0055] The term “extracorporeal device” refers to an instrument that receives blood that is removed from a patient, processes (e.g., filters, purifies, treats, administers therapeutic agents to, etc.) the blood, and then returns the blood to the patient.

[0056] The term “magnetic particles” includes beads, microbeads, and particles that a magnetic or paramagnetic.

[0057] The term “individual,” “subject,” or “patient” typically refers to humans, but also to other animals including, e.g., other primates, rodents, canines, felines, equines, ovines, porcines, and the like.

III. Detailed Descriptions of Embodiments

[0058] The methods provided herein can be used to treat a cancer patient post tumor resection (e.g., tumor excision by surgery) or treatment by reducing the circulating tumor cells (CTCs) in the blood, and thus, reducing the chance of metastatic spread. Photodynamic therapy (PDT) utilizes photo-
sensitizers (e.g., photosensitizing agents or drugs) and light exposure to kill cancer cells. For example, the photosensitizing agent, porlimer sodium (e.g., Photofrin®) is FDA approved for use in PDT to treat the symptoms of esophageal cancer and non-small cell lung cancer. Aminolevulinic acid (ALA or Levalun®) and methyl ester of ALA (Metvix® cream) are FDA approved for skin or topical treatment for PDT. Most photosensitizing agents are not targeted and require irradiation of the whole body or a large region, which can result in unwanted cell death of normal tissues.

[0060] Provided herein is a treatment method using an extracorporeal device that can circulate blood containing CTC targeted agents and to either induce CTC cell death (e.g., apoptosis, necrosis or autophagy) or to separate the CTCs from the blood returning to the body. The cell death is induced by photodynamic therapy method for CTCs that employs an IRDye®-targeting agent that can be excited by an irradiating light of a wavelength from 660 nm to about 740 nm. The irradiated blood can be returned (e.g., reintroduced) to the subject where the subject’s immune system can recognize the apoptotic and/or necrotic cells and target them for removal. In some embodiments, the extracorporeal device is to circulate blood outside the body for the treatment of the agent targeted CTCs.

[0061] A. Phthalocyanine Dye Conjugated to a Targeting Moiety

[0062] Disclosed herein are targeted photosensitizing agents such as IRDye® 700DX-CTC moieties. Any targeting moiety (e.g., an antibody or fragment thereof, nanobody, Affibody®, diabody, minibody, antigen, ligand, protein, peptide, carbohydrate, nucleic acids, or small molecules) that specifically binds to a circulating tumor cell can be used.

[0063] In some embodiments, the targeting moiety is specific for a protein expressed on the cell surface of a CTC. The cell surface protein can be selectively expressed on the surface of CTCs, and not other cells such as normal cells. In some instances, the cell surface protein is epithelial cell adhesion molecule (EpCAM), cytokeratin 8, cytokeratin 18, cytokeratin 19, E-cadherin, fibronectin, vimentin, MUC1, Her2, Twist, E-selectin, HSPG, CD44, osteocyte, and others.

[0064] One skilled in the art recognizes that cell surface protein sequences are publicly available and that making or purchasing antibodies specific for such proteins are routine. For example, EpCAM antibodies are available from, e.g., Abcam® (Cambridge, Mass.), Cell Signaling Technology® (Danvers, Mass.), Santa Cruz Biotechnology® (Santa Cruz, Calif.), and other companies.

[0065] The generation and selection of antibodies are not commercially available for targeting circulating tumor cells in accordance with the present invention can be accomplished several ways. For example, one way is to express and/or purify a polypeptide of interest (i.e., antigen) using protein expression and purification methods known in the art, while another way is to synthesize the polypeptide of interest using solid phase peptide synthesis methods known in the art. See, e.g., Guide to Protein Purification, Murray P. Deutche, ed., Meth. Enzymol., Vol. 182 (1990); Solid Phase Peptide Synthesis, Greg B. Fields, ed., Meth. Enzymol., Vol. 289 (1997); Kiso et al., Chem. Pharm. Bull., 38:1192-99 (1990); Mostafavi et al., Biomed. Pept. Proteins Nucleic Acids, 1:255-60, (1995); and Fujiiwara et al., Chem. Pharm. Bull., 44:1326-31 (1996). The purified or synthesized polypeptide can then be injected, for example, into mice or rabbits, to generate polyclonal or monoclonal antibodies. One skilled in the art will recognize that many procedures are available for the production of antibodies, for example, as described in Antibodies, A Laboratory Manual, Harlow and Lane, Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988). One skilled in the art will also appreciate that binding fragments or Fab fragments which mimic (e.g., retain the functional binding regions of) antibodies can also be prepared from genetic information by various procedures. See, e.g., Antibody Engineering: A Practical Approach, Borresch, Ed., Oxford University Press, Oxford (1995); and Huse et al., J. Immunol., 149:3914-3920 (1992).

[0066] Conjugation of IRDye® 700DX-CTC Targeting Moiety

[0067] The CTC targeting agent (e.g., CTC targeting moiety) can be conjugated to a phthalocyanine dye, IRDye® 700DX (LI-COR, Lincoln, Nebr.). In some embodiments, CTC targeting moieties are labeled with IRDye® 700DX according to the manufacturer’s protocols and kits. Detailed descriptions of methods for producing IRDye® 700DX-conjugates are found in, e.g., Kavai et al., Biochemistry—Faculty Publications, 2007, paper 9; Mitsuhashi et al., Nature Medicine, 2011, 17:1665-1691; Peng et al., Proceedings of SPIE, 2006, 6097; U.S. Pat. Nos. 7,005,518 and 8,524,239; and U.S. Patent Application Publication No. 2013/0336995, the disclosures of each are herein incorporated in their entirety for all purposes.

[0068] The IRDye® 700DX-CTC moiety can be administered to the subject’s blood in the blood circuit of the extracorporeal device. For instance, the targeting agent is injected into the blood that has exited the subject prior to treatment by the excitation light.

[0069] In some embodiments, the dose of IRDye® 700DX-CTC moiety or biomolecule can be removed or cleared from the blood before returning to the subject. For instance, the removal or clearance methods can be mechanisms involved in molecular movement or attachment to isolate the agent from the blood stream so that the returning blood contains minimal amount of the therapeutic agent.

[0070] In some embodiments, the IRDye® 700DX-CTC moiety or biomolecule can be administered systemically using any method known in the art, for example to subjects having a tumor, such as a cancer, or who has had tumor resection (e.g., tumor removal, for example, by surgery). One skilled in the art will appreciate that any method of systemic administration of the disclosed IRDye® 700DX-CTC moiety or biomolecule can be used. Such methods may include for example, the use of catheters or implantable pumps to provide continuous infusion over a period of several hours to several days into the subject in need of treatment. Other methods include direct injection or infusion into the blood. Alternatively, the IRDye® 700DX-CTC moiety or biomolecule can be administered systemically, for example intravenously, intramuscularly, subcutaneously, intradermally, intraperitoneally, subcutaneously, or orally, to a subject having CTCs.

[0071] The dosages of IRDye® 700DX-CTC moiety to be administered to a subject will depend on the nature of the composition and its active ingredients and its unwanted side effects (e.g., immune response against the moiety or biomolecule), the subject being treated and the type of condition being treated and the manner of administration. Generally the dose will be a therapeutically effective amount, such as an amount sufficient to achieve a desired biological effect, for
example an amount that is effective to reduce the number of CTCs in the subject, eliminate the CTCs, or decrease the chance of metastasis spread.

[0072] For intravenous administration of the IRDye® 700DX-CTC moiety or biomolecule, exemplary dosages for administration to a subject for a single treatment can range from about 0.5 to 100 mg/60 kg of body weight, about 1 to 100 mg/60 kg of body weight, about 1 to 50 mg/60 kg of body weight, about 1 to 20 mg/60 kg of body weight, for example about 1 or 5 mg/60 kg of body weight.

[0073] Treatments with the IRDye® 700 DX-CTC moiety or biomolecule can be completed in a single day, or may be done repeatedly on multiple days with the same or a different dosage. Repeated treatments may be done on the same day, on successive days, or every 1-3 days, every 3-7 days, every 1-2 weeks, every 2-4 weeks, every 1-2 months, or at even longer intervals.

[0074] B. Irradiation of Targeted CTCs

[0075] After the cells are contacted with one or more IRDye®700DX-CTC moiety or biomolecule, they are removed from the patient’s body using an extracorporeal device wherein they are irradiated. In some embodiments, blood containing the targeted cells is removed from the patient using a dialysis-like device and exposed to an irradiating light, e.g., at a wavelength of 660-740 nm.

[0076] In some embodiments, the cells in the blood are irradiated with a therapeutic dose of radiation at a wavelength of about 660-740 nm, e.g., about 660-700 nm, 670-690 nm, 660-680 nm. In preferred embodiments, the cells are irradiated with a therapeutic dose of radiation at a wavelength of about 680 nm. In some embodiments, the cells are irradiated at a dose of at least 1 J cm⁻², e.g., at least 1 J cm⁻², at least 10 J cm⁻², at least 30 J cm⁻², at least 50 J cm⁻², at least 100 J cm⁻², at least 500 J cm⁻². In other embodiments, the irradiating light is applied at, for example, 1-1000 J cm⁻², 1-500 J cm⁻², 10-100 J cm⁻², or 10-50 J cm⁻².

[0077] The targeted cells in the blood can be irradiated one or more times. In some embodiments, irradiation can be completed in a single day, or may be done repeatedly on multiple days with the same or a different dosage (such as irradiation at least 2 different times, 3 different times, 4 different times, 5 different times or 10 different times). Repeated irradiations may be done on the same day, on successive days, or every 1-3 days, every 2-4 weeks, every 1-2 months, or at even longer intervals.

[0078] C. Extracorporeal Photodynamic Therapy Device

[0079] An exemplary embodiment of the method and the device 100 is provided in FIG. 1.

[0080] The method and device provided herein can be used for performing extracorporeal CTC photodynamic therapy on a patient with circulating tumor cells using an extracorporeal circuit 110, and a radiation chamber 150 housed in or separately from the extracorporeal device 100. Blood is drawn from the patient, bearing an intravenous catheter, an arteriovenous fistula (AV) or a synthetic graft, etc., through the blood line 120 and transported to the fluid circuit 110. A pump system 130 comprising pumps (e.g., peristaltic pumps) and tubing for the transport of flow from and to different unit sections. In some embodiments, the blood pressure, temperature, pH, and/or other parameters are monitored. An anticoagulant can be added via, for example, injection to the blood in the circuit to prevent blood clotting. In some embodiments, the IRDye® 700-CTC targeting agent (e.g., IRDye® 700DX-CTC moiety or biomolecule) is added to the blood via the agent administration unit 140 with controlled flow rate and/or flow pressure. In some embodiments, the blood including the IRDye® 700DX labeled CTCs enter a phototherapy chamber 150 where the blood is exposed to irradiating light in the illumination circuit. The treated blood exits the phototherapy chamber 150 and is moving toward blood reintroducing line 121. In some embodiments, the therapeutic agent (e.g., the unbound therapeutic agent) is removed or reduced via the agent clearance chamber 170 before returning the body from the blood reintroducing line 121. The clearance chamber can be a dialyzer, a supernatant remover, a filter, or other physical methods for the clearance of the agent molecules.

[0081] Within the phototherapy chamber 150 a light source provides an excitation light at a wavelength of about 660 nm to about 740 nm to the blood and labeled target CTCs as they pass through the phototherapy chamber. Exposure to the irradiating light induces the CTCs to undergo cell death, such as apoptosis, necrosis or autophagy.

[0082] D. Extracorporeal CTC Removal Device

[0083] Another exemplary embodiment of the method and the device 200 is provided in FIG. 2.

[0084] The methods herein can be used for performing extracorporeal CTC removal with the extracorporeal circuit 210 to reduce the number of CTCs before reintroducing the cleaned blood to the body. Blood is drawn from the patient through blood line 220 and transported to the extracorporeal circuit 210. A pump system 230 comprising pump(s) (e.g., peristaltic pumps) and tubing for the transport of flow from and to different unit sections. In some embodiments, the blood pressure, temperature, pH, and/or other parameters can be monitored. Chosen anticoagulant can be added via injection to the blood in the circuit to prevent blood clotting. In some embodiments, the magnetic particle targeting agent (e.g., magnetic beads-CTC moiety) is added to the blood via the agent administration unit 240 with controlled flow rate and/or flow pressure. In some embodiments, the blood including the targeted CTCs enter a magnetic influence chamber 250 where magnetic field is applied to the flow circuit. The magnetic field retains magnetic particle targeted CTCs from the blood flow before returning to the body through the reintroducing line 221. In some embodiments, the agent remnants (e.g., the unbound agents) are removed or reduced via the agent clearance chamber 270. The clearance chamber can be a filter, a dialyzer, a supernatant remover, or other methods for the clearance of agent molecules.

IV. Examples

[0085] The following examples are offered to illustrate, but not to limit, the claimed invention.

Example 1

Using IRDye® 700DX-EGF Probe and Photoactivating Light to Kill Cancer Cells Overexpressing the EGF Receptor

[0086] This example illustrates the application of photodynamic therapy using an IRDye® 700DX-targeting agent (e.g., EGF-700DX) and photoactivating light to kill targeted cells.

[0087] Cancer cells that overexpress the EGF receptor (e.g., A431 cells) were seeded in an in vitro culture system. The cells were incubated with the targeting agent EGF-
700DX and exposed to irradiating light at a wavelength of 690 nm and at 8 J/cm² or 16 J/cm².

**[0088]** FIG. 3 shows a reduction in the number of targeted cells after exposure to irradiating light at 8 J/cm² or 16 J/cm². FIGS. 3A-E show a reduction in the number of targeted cancer cells after exposure to irradiating light at 8 J/cm² or 16 J/cm². FIGS. 3A-C are control data and FIGS. 3D-E show exposure to 8 J/cm² or 16 J/cm², respectively. In addition, FIG. 3E shows significant morphology changes in cells treated with EGF-700DX and irradiating light at 16 J/cm².

**[0089]** To determine the effect of whole blood on the photodynamic method, cultured cancer cells were incubated with EGF-700DX. Blood was added to the culture dish to form a 1 mm layer on top of the cells but not in direct contact with them. The blood/cell chamber was exposed to irradiating light at a wavelength of 690 nm at 16 J/cm². The photodynamic reaction between the EGF-700DX and the light occurred in the presence of the blood. There was a decrease in cell viability in the cells treated with irradiating light (e.g., 42% cell viability compared with 98.0-96.6% for controls).

**[0090]** FIG. 4 shows a reduction in the number of targeted cells in blood after exposure to irradiating light at 670 nm to 700 nm at 8 J/cm² or 16 J/cm². FIGS. 4A-D show a reduction in the number of targeted cancer cells in blood after exposure to irradiating light at 670 nm to 700 nm at 8 J/cm² or 16 J/cm². FIGS. 4A-B are control data and FIGS. 4C-D show exposure to 8 J/cm² or 16 J/cm², respectively.

**[0091]** To determine the effect of flow rate and time on the ability of EGF-700DX to bind cultured cells (A431), the targeting conjugate was flowed over the cells in a closed perfusible pump setup at two different concentrations for two different time periods. Binding was evaluated at this step in the testing phase. No irradiation was added at this point.

**[0092]** FIGS. 5A and 5B show that EGF-700DX successfully bound to the cultured cells. The flow rate of the conjugate concentration was 1.36 mL/min. A431 cells received either EGF-700DX (0.2 μM) for 15 min (FIG. 5A) or EGF-700DX (0.5 μM) for 5 min (FIG. 5B).

**[0093]** In summary, this example shows that cancer cells were killed by labeling them with an IRDye® 700DX-targeting agent (e.g., EGF-700DX) and exposing the labeled cells to phototracivating light. Furthermore, the presence of blood with the labeled cells did not affect the photodynamic method of irradiation. Additionally, the results illustrate that the IRDye® 700DX-targeting agent effectively binds to stationary cells within minutes.

**Example 2**

Methods for Inducing Apoptosis, Necrosis or Autophagy in Cancer Cells Using an IRDye® 700DX-EGF® Probe and Photocatalyzing Light

**[0094]** A study was performed to optimize the conditions for IRDye® 700DX-EGF® based photodynamic therapy. In the experiment factors (e.g., parameters and variables) that contribute to the level of apoptosis and/or necrosis are tested. In particular, three factors with two conditions for each were evaluated in a 2×2×2 factorial design of experiments. The factors included: A) probe concentration at 0.5 μM and 2.0 μM, B) irradiation of 4 J/cm² or 16 J/cm², and C) probe incubation time of 5 minutes or 10 minutes. All possible treatment conditions were tested. Representative images of the treated cells are provided in FIGS. 6A-D and 7A-7D.

**[0095]** Briefly, cancer cells that overexpress EGF (e.g., A431 cells) were plated and incubated with the IRDye® 700DX-EGF® probe at the test concentration and at a flow rate of 1.36 mL/min. After the incubation, the cells were washed to remove any unbound probe prior to irradiation. Afterwards, images of the cells were captured to determine the location of the probe and the extent of probe binding. The cells were then irradiated and imaged to identify morphology changes. 24 hours after irradiation, cell vitality (e.g., cell count and cell viability) was assessed using the NucleoCounter® NC-300™ assay (Chemometec, Allerod, Denmark).

**[0096]** The data shows that the IRDye® 700DX-EGF® probe effectively bound to the cell surface of the EGF-positive cells after a brief incubation period. After incubation for 5 minutes with 0.5 μM probe (FIG. 6A) or 2.0 μM probe (FIG. 6C), the cells were irradiated at 4 J/cm² (FIGS. 6B and 6D, respectively). No visual difference was observed between the treatments.

**[0097]** In the next set of conditions, the cells were incubated for 10 minutes with either 0.5 μM probe (FIG. 7A) or 2.0 μM probe (FIG. 7C) and irradiated at 16 J/cm² (FIGS. 7B and 7D, respectively). Some change in morphology was detected immediately after irradiation. Apoptosis and/or necrosis was assessed 24 hours later using the cell vitality assay. The data shows that cell death was higher (e.g., lower cell viability) for treatments conducted at 16 J/cm², such as for treatments 3, 4, 7, and 8 (FIG. 8).

**[0098]** Statistical analysis was performed to determine if data transformation was necessary. The Box-Cox plot was generated that showed that transformation was not required (FIG. 9A). The normal plot of residuals showed that the data fit into a straight line, thereby indicating that the residuals followed a normal distribution and were a good fit (FIG. 9B). In addition, ANOVA information was obtained for the 2×2×2 factorial experiment (FIG. 9C). The results demonstrate that the irradiation parameters had a significant effect on cell vitality. The other factors, such as the test probe concentrations and probe incubation times did not affect apoptosis and/or necrosis.

**[0099]** In summary, this example shows the use of a photodynamic therapeutic method for initiating cell death in cancer cells expressing the EGF receptor. The experiments showed that the IRDye® 700DX-EGF® probe selectively targeted the cancer cells which were then irradiated by a phototracivating light. The cell vitality assay showed that some of the treated cells underwent cell death.

**Example 3**

Methods for Inducing Apoptosis, Necrosis or Autophagy in Cancer Cells Using an IRDye® 700DX-EGF® Probe or an IRDye® 700DX-Panitumumab Probe

**[1000]** This example illustrates that the IRDye® 700DX-EGF® probe and the IRDye® 700DX-pantuctumab probe can induce cell death in A431 cells which are human epithelial cancer cells that overexpress the EGF receptor. EGF is the ligand for the EGF receptor and pantumumab is a fully human monoclonal antibody specific to the receptor.

**[1001]** A431 cells were plated and incubated with either probe for 5 minutes, 10 minutes or 20 minutes prior to washing and irradiation at 24 J/cm². The IRDye® 700DX-EGF® probe was used at a concentration of 0.5 μM in complete media and the IRDye® 700DX-pantumumab probe was
used at a concentration of 0.1 μM. After irradiation, the cells were cultured at 37°C, 5% CO₂ for approximately 24 hours. The NucleoCounter® NC-3000™ cell vitality assay (Chemo-meter, Allerød, Denmark) was performed. Table 1 provides cell numbers. Table 2 provides percent cell vitality.

### TABLE 1

<table>
<thead>
<tr>
<th>Cell Number (cells/mL)</th>
<th>5 minutes</th>
<th>10 minutes</th>
<th>20 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye® 700DX-EGF</td>
<td>1,000,000</td>
<td>1,000,000</td>
<td>1,500,000</td>
</tr>
<tr>
<td>IRDye® 700DX-panitumumab</td>
<td>675,000</td>
<td>323,000</td>
<td>550,000</td>
</tr>
</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Cell Vitality (%)</th>
<th>5 minutes</th>
<th>10 minutes</th>
<th>20 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye® 700DX-EGF</td>
<td>83.9%</td>
<td>85.2%</td>
<td>92.3%</td>
</tr>
<tr>
<td>IRDye® 700DX-panitumumab</td>
<td>44.2%</td>
<td>34%</td>
<td>31%</td>
</tr>
</tbody>
</table>

[0102] The data show a significant difference in the effectiveness of the probes at these concentrations to induce cell death (Table 2 and FIG. 10). The IRDye® 700DX-panitumumab averaged about 60% cell death and the IRDye® 700DX-EGF averaged about 20% over the time periods. The longer incubation time (e.g., 20 minutes vs. 5 minutes) with the antibody probe increased the number of cells undergoing cell death. The cell morphology also indicated that the treated cells were undergoing apoptosis, necrosis or autophagy (FIGS. 11A–11C). Blebs which are typical of cells in apoptosis or necrosis were observed on the cell surface of the targeted cells.

[0103] This examples illustrates that IRDye® 700DX targeting probes, such as those based on proteins and antibodies, were able to target select cancer cells. The probe-bound cells underwent induced cell death upon photoactivation of the probe.

[0104] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.

What is claimed is:

1. A method for inducing apoptosis and/or necrosis resulting in cell death of circulating tumor cells (CTCs) in a subject, the method comprising:
   (a) administering to the subject’s blood a therapeutically effective agent comprising a phthalocyanine dye conjugated to a CTC targeting moiety that specifically binds to CTCs;
   (b) ex vivo irradiating the subject’s blood with an appropriate excitation light in an amount to effectively induce apoptosis, necrosis or autophagy in the CTCs; and
   (c) re-introducing the irradiated blood into the subject.
2. The method of claim 1, wherein the phthalocyanine dye is IRDye® 700DX.
3. The method of claim 1, wherein the targeting moiety is selected from the group consisting of an antibody or fragment thereof, nanobody, Affibody®, diabody, minibody, antigen, ligand, protein, peptide, carbohydrate, nucleic acids, and small molecule.
4. The method of claim 1, wherein the targeting moiety specifically binds a CTC antigen.
5. The method of claim 1, wherein the therapeutically effective agent is administered with an anticoagulant.
6. The method of claim 1, wherein ex vivo irradiating is performed using an extracorporeal device.
7. The method of claim 1, further comprising after step (a) removing the unbound therapeutically effective agent from the subject’s blood by filtration or dialyzing.
8. A method of extracorporeal therapy for treating cancer in a subject having circulating tumor cells (CTCs), the method comprising:
   (a) intravenously administering a therapeutically effective agent comprising a phthalocyanine dye conjugated to a CTC targeting moiety that specifically binds to CTCs;
   (b) ex vivo irradiating the subject’s blood with an appropriate excitation light in an amount to effectively induce apoptosis, necrosis or autophagy in the CTCs; and
   (c) re-introducing the irradiated blood into the subject, thereby treating cancer in the subject.
9. The method of claim 8, wherein the subject has undergone solid tumor resection.
10. The method of claim 8, wherein the phthalocyanine dye is IRDye® 700DX.
11. The method of claim 8, wherein the targeting moiety is selected from the group consisting of an antibody or fragment thereof, nanobody, Affibody®, diabody, minibody, antigen, ligand, protein, peptide, carbohydrate, nucleic acids, and small molecule.
12. The method of claim 8, wherein the targeting moiety specifically binds a CTC antigen.
13. The method of claim 8, wherein the therapeutically effective agent is administered with an anticoagulant.
14. The method of claim 8, wherein step (b) and/or (c) are performed using an extracorporeal device.
15. The method of claim 8, further comprising after step (a) removing the unbound therapeutically effective agent from the subject’s blood by filtration or dialyzing.
16. A method of extracorporeal treatment of cancer in a subject having circulating tumor cells (CTCs), the method comprising:
   (a) administering to the subject a CTC targeting agent comprising magnetic particles conjugated to a targeting moiety that specifically binds to CTCs, wherein the targeting agent and the CTCs form a complex;
   (b) ex vivo separating the blood from the complex using a magnetic field in an extracorporeal device; and
   (c) re-introducing the blood into the subject, thereby treating cancer in the subject.
17. The method of claim 16, wherein the targeting agent is introduced ex vivo by the extracorporeal device.
18. The method of claim 16, wherein step (a) is performed using the extracorporeal device.
19. The method of claim 16, wherein the subject has undergone solid tumor resection.
20. The method of claim 16, wherein the targeting moiety specifically binds a CTC antigen.

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