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***PROTEOLYSIS-TARGETING CHIMERA TUMOR VACCINE***

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**RELATED APPLICATION**

This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/538,122, filed September 13, 2023; the contents of which are hereby incorporated by reference in their entirety.

**BACKGROUND**

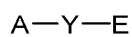
Cytotoxic T cells initiate robust adaptive immune responses via recognizing peptide-major histocompatibility complex (MHC) I complexes on the surface of antigen presenting cells (APCs). For vaccination-based cancer immunotherapy, tumor antigen processing and MHC-I cross-presentation are crucial steps. Specifically, antigens in cancer vaccines must be 1) endocytosed by APCs, 2) trafficked to the cytoplasm from endo-lysosomes, and 3) degraded into short peptides (approximately 8–13 amino acids) via the ubiquitin-proteasome pathway (UPP). These peptide fragments are subsequently loaded onto MHC-I binding grooves and located to the APC surface for cross-priming. Strategies using cell-penetrating peptides, ‘proton sponge’ polymers, and pore-formation molecules have been developed to facilitate endo-lysosomal escape of tumor antigens to boost their immunogenicity. However, development of highly efficient anti-tumor vaccines is still a challenge due to limited cytosolic transport efficiency. Improving endo-lysosomal escape can increase the cytosolic amount of tumor antigens to amplify MHC-I pathway-mediated cross-presentation. This also increases the risk of digestive enzymes leaking from endo-lysosomes, which cause cell necrosis. Thus, maximizing the conversion of escaped tumor antigens into antigenic peptides is expected to enhance anti-tumor activity, but is seldom investigated.

In UPP-mediated protein degradation, a rate-limiting step is the recruitment of E3 ubiquitin ligases to a protein of interest (POI). However, degradation of tumor antigens to antigenic peptides via UPP is not an efficient process due to the lack of a mechanism by which escaped tumor antigens bind to E3 ubiquitin ligases. Additionally, the macromolecular crowding effect within living cells further reduces the probability of collisions between tumor antigens and E3 ubiquitin ligases.

### SUMMARY OF THE INVENTION

Cytotoxic T-cell activation typically requires the presentation of an epitope derived from tumor antigen in MHC class I expressed by antigen presenting cells (APCs). Ubiquitin–proteasome pathway (UPP)-mediated proteolysis (degradation) of tumor antigens, namely, antigen processing, is a critical step. Directing tumor antigens to target the UPP could be an alternative route to augment antigen processing and cross-presentation. Disclosed herein are targeted antigen degradation-based tumor vaccines (TAgD-TVac) using tumor antigens pre-fused with proteolysis targeting chimera (PROTAC). When delivered using a lymph node-targeting lipid nanoparticle, the PROTAC conjugation targets tumor antigens to the UPP under the guidance of E3 ligase ligands in APCs, which amplifies antigen cross-presentation and initiates robust cytotoxic T-cell activation. It is demonstrated that the TAgD-TVacs prepared with relevant tumor lysate elicit potent antigen-specific adaptive immunity and immunological memory against multiple solid tumors, including melanoma, triple-negative breast cancer, Lewis lung carcinoma, and (clinically inoperable) ovarian cancer, demonstrating its clinical translational potential.

In one aspect, the present disclosure provides compounds having a structure represented by formula I or a pharmaceutically acceptable salt thereof:



I

wherein,

A is a tumor antigen;

E is an active agent (e.g., a drug); and

Y is a linker that covalently links A to E and comprises an alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene, arylene, or heteroarylene, or a combination thereof, wherein the linker comprises 1-100 carbon atoms, preferably 10-50 carbon atoms.

In another aspect, the present disclosure provides nanoparticles comprising a tumor antigen covalently linked to an active agent; and a plurality of lipids or lipidoids.

In another aspect, the present disclosure provides pharmaceutical compositions comprising a nanoparticle disclosed herein; and a pharmaceutically acceptable excipient.

In another aspect, the present disclosure provides methods of treating cancer in a subject in need thereof comprising administering a therapeutically effective amount of the compounds or nanoparticles disclosed herein.

In another aspect, the present disclosure provides methods of treating an infectious disease in a subject in need thereof comprising administering a therapeutically effective amount of the compounds or nanoparticles disclosed herein.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1** shows a schematic for a targeted antigen degradation-based tumor vaccine (TAgD-TVac) enhances antigen cross-presentation and induces robust anti-tumor immune response. TAgD-TVac is prepared by loading pre-fused PROTAC tumor antigens to an adjuvant lipid nanoparticle (LNP) with lymph node (LN)-targeting capability. Pre-fused PROTAC tumor antigen is obtained by covalently modifying (*S,R,S*)-AHPC onto a tumor antigen. After being taken up by antigen presenting cells (APCs), pre-fused PROTAC tumor antigens are released from LNPs and bind to E3 ubiquitin ligases, thereby undergoing ubiquitination and proteasome-mediated antigen degradation. The resulting tumor antigenic peptides bind to the major histocompatibility complex class I (MHC-I), inducing robust cytotoxic T-cell cross-priming. TAgD-TVac shows potent anti-tumor therapeutic outcomes in (subcutaneous) breast cancer, (orthotopic) Lewis lung carcinoma, and (inoperable) ovarian cancer.

**FIGs. 2A-2K** show the preparation of TAgD-TVac and the enhanced cross-presentation in DCs by improving proteasome-mediated antigen degradation. **FIG. 2A** shows the preparation of the pre-fused PROTAC tumor antigen by using 2-aminothiophene (Traut's reagent) and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) as linkers. The antigens are modified with Traut's reagent, followed by reacting with maleimidated analogue of (*S,R,S*)-AHPC. **FIG. 2B** shows fluorescence images of the mixture of pre-fused AHPC-GFP-Cre (green) and von Hippel-Lindau protein (pVHL, red). pVHL is pre-labelled with RhB. Pre-fused AHPC-GFP-Cre and pVHL were mixed in a molar ratio of 1.5:1, then incubated at 37 °C for 2 hours. Scale bar, 5 μm. **FIG. 2C** shows pVHLs are immobilized onto the surface of PLGA NPs to evaluate the interactions between pre-fused AHPC-GFP-Cre and pVHL. **FIG. 2D** shows agarose gel electrophoresis results of pre-fused AHPC-GFP-Cre after incubating with pVHL-modified PLGA NPs. The mixture of GFP-Cre, AHPC, and pVHL-modified PLGA NPs is used as a control. **FIG. 2E** shows the chemical structure of adjuvant (113-O12B) lipidoid. **FIG. 2F** shows representative transmission electron microscopy (TEM) images of adjuvant LNP and TAgD-TVac. Scale bar, 200 nm. **FIG. 2G** shows representative immunofluorescence images of DC2.4 cells after incubating with GFP-Cre + AHPC LNPs and pre-fused AHPC-GFP-Cre LNPs for 12 hours, respectively.

LNP/protein = 10/1, w/w. Pre-fused AHPC-GFP-Cre emits green fluorescence. pVHLs are stained by anti-VHL antibodies, followed by staining with fluorescent secondary antibodies. Scale bar, 10  $\mu$ m. **FIG. 2H** shows the degradation kinetics of GFP-Cre in DC2.4 cells after incubation with different LNP formulations for 3 hours. GFP-Cre fluorescence is monitored over 24 hours. **FIG. 2I** shows Western blotting analysis of cellular OVA levels in bone marrow-derived dendritic cells (BMDCs) after incubating with different LNP formulations for 3 hours. Cellular OVA levels are evaluated after further 24-hour incubation. LNP/protein = 10/1, w/w. **FIGS. 2J & 2K** show representative flow cytometry analysis and statistic analysis of the expression of H2kb-SIINFEKL in BMDCs after different treatments *in vitro*. Data are presented as mean  $\pm$  standard deviation (s.d.) from *n* independent experiments (*n* = 3). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test. (\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

**FIGS. 3A-3R** show that TAGD-TVac<sub>OVA</sub> targets lymph nodes (LNs) and induces robust anti-tumor immunity *in vivo*. **FIG. 3A** shows representative IVIS images of fluorescence distribution in BALB/c mice after subcutaneous (SC) injection of Cy5.5-labelled ALC-0315<sub>OVA</sub> and TAGD-TVac<sub>OVA</sub> for 6 hours. Pre-fused AHPC-OVA is pre-labelled with Cy5.5. **FIG. 3B** shows representative *ex vivo* fluorescence images of the main organs. **FIG. 3C** shows the ratio of radiance in inguinal LNs and liver. **FIG. 3D** shows that TAGD-TVac<sub>OVA</sub> reaches the LNs via afferent lymphatic vessels. **FIG. 3E & 3F** show Western blotting analysis of the levels of OVA in LNs after administration with different LNP formulations. A mixture of OVA and the tissue lysate of LNs is used as the positive control (PC). **FIG. 3G** shows the mechanism of subcellular analysis of Cre-mediated gene recombination in Ai14 mice. The gene encoding tdTomato is blocked by a stop gene between two loxP segments. The loxP gene will be cut when Cre recombinases are delivered into the cytoplasm. The tdTomato gene is subsequently activated, and the cells express tdTomato with red fluorescence. **FIG. 3H** shows the percentage of tdTomato-positive (tdTomato<sup>+</sup>) cells in different types of immunocytes after SC injection of GFP-Cre-loaded adjuvant LNPs. Naked GFP-Cre is used as a control. **FIG. 3I** shows Clodrosome® injected to the mice to deplete macrophages. The mice are subcutaneously treated with Clodrosome® 18 hours before administration of Cy5.5-labelled TAGD-TVac<sub>OVA</sub>. Clodrosome® is a well-characterized liposomal drug that has been used to deplete macrophages either in local tissues or peripheral blood, which depends on the injection routes. **FIG. 3J** shows *Ex vivo* fluorescence images of the isolated LNs at 6 hour after injection of Cy5.5-labelled TAGD-TVac<sub>OVA</sub>. **FIG. 3K** shows a schematic illustration of the timeline for B16F10-OVA tumor inoculation and vaccination in a C57BL/6 mouse model. B16F10-OVA

cells ( $1 \times 10^6$  per mouse) are subcutaneously injected at the left flank of C57BL/6 mice. **FIGS. 3L & 3M** show individual tumor growth curves and average tumor growth kinetics after administration with different LNP formulations. **FIG. 3N** shows survival of B16F10-OVA tumor-bearing mice. **FIG. 3O** shows flow cytometry analysis of the expression of H2kb-SIINFEKL in DCs (gated on CD11C<sup>+</sup> cells) within LNs after different treatments. **FIG. 3P** shows flow cytometry analysis of the population of CD8<sup>+</sup> T-cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 3Q** shows flow cytometry analysis of the expression of GzmB<sup>high</sup> in CD8<sup>+</sup> T-cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 3R** shows flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> T-cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments (c, f, h, o, p, q, and r,  $n = 3$ ; j,  $n = 4$ ; m, n,  $n = 6$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test for c, f, o, p, q, and r, two-way ANOVA with Tukey's test for h and m, log-rank (Mantel–Cox) test for n, and t test for c and j. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIGS. 4A-4L** shows that TAgD-TVac<sub>4T1</sub> induces robust anti-tumor immunity and inhibits lung metastasis in a breast cancer model. **FIG. 4A** shows a schematic illustration of the timeline for 4T1-Luc tumor inoculation and vaccination in a BALB/c mouse model. 4T1-Luc tumor cells ( $1 \times 10^6$  per mouse) were subcutaneously injected at the left flank of the mice. **FIG. 4B** shows *in vivo* bioluminescence imaging of 4T1-Luc tumor growth after administration with different LNP formulations. **FIGS. 4C & 4D** show individual tumor growth curves and average tumor growth kinetics. CR, complete response. **FIG. 4E** shows the survival of 4T1-Luc tumor-bearing mice. **FIG. 4F** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs after different treatments. **FIG. 4G** shows flow cytometry analysis of the population of CD8<sup>+</sup> T-cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 4H** shows flow cytometry analysis of the expression of GzmB<sup>high</sup> in CD8<sup>+</sup> T-cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 4I** shows flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> T-cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. **FIG. 4J** shows a schematic illustration of the design of tumor rechallenge experiment. The mice after different treatments are rechallenged with 4T1-Luc cells ( $1 \times 10^6$  per mouse) via IV injection on day 18. **FIGS. 4K & 4L** show Bioluminescence images of the mice and lungs after rechallenging with 4T1-Luc tumor cells. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments (d, e,  $n = 6$ ; f, g, h, i,  $n = 3$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test for f, g, h and i, two-

way ANOVA with Tukey's test for d, and log-rank (Mantel–Cox) test for e. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIGs. 5A-5M** shows the results of TAgD-TVac<sub>LLC</sub> combined with ICB therapy for orthotopic Lewis lung cancer. **FIG. 5A** shows a schematic illustration of the timeline for LLC-Luc tumor inoculation ( $0.5 \times 10^6$  per mouse) and treatments in a C57BL/6 mouse model. **FIG. 5B** shows *In vivo* bioluminescence imaging of LLC-Luc tumor growth after administration with different formulations. **FIGs. 5C & 5D** show *ex vivo* bioluminescence imaging and photographs of the lungs collected from different treatment groups (taken with iphone 14 Pro Max). **FIG. 5E** show the body weight of LLC-Luc tumor-bearing mice at different time points. Mice are euthanized when they exhibit >15% loss of baseline body weight, which reach the humane endpoint. **FIG. 5F** shows the survival of LLC-Luc tumor-bearing mice. **FIG. 5G** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs after different treatments. **FIG. 5H** shows IL-6 levels in the blood. **FIG. 5I** shows flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> T-cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. **FIG. 5J** shows a schematic illustration of the design of tumor rechallenge experiment. **FIG. 5K** shows that LLC-Luc cells ( $1 \times 10^6$  per mouse) were subcutaneously injected at the left flank of C57BL/6 mice, and B16F10 tumor cells ( $1 \times 10^6$  per mouse) were inoculated in the opposite flank of the LLC-Luc tumors. **FIG. 5L** shows photographs of LLC-Luc tumors and B16F10 tumors. **FIG. 5M** shows average tumor growth kinetics. TFS, tumor-free survival. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments (e, f,  $n = 6$ ; g, h, i,  $n = 3$ ; m = 5). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test for g, h and i, two-way ANOVA with Tukey's test for e and m, and log-rank (Mantel–Cox) test for f. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIGs. 6A-6O** show the results of TAgD-TVac<sub>ID8</sub> combined with ICB therapy for inoperable ovarian cancer. **FIG. 6A** shows a schematic illustration of the timeline for ID8-Luc tumor inoculation and treatments in a C57BL/6 mouse model. ID8-Luc ( $1 \times 10^7$  per mouse) tumor cells were injected intraperitoneally into female C57BL/6 mice for the establishment for inoperable ovarian cancer model. **FIG. 6B** shows *in vivo* bioluminescence imaging of ID8-Luc tumor growth after administration with different formulations. **FIG. 6C** shows *ex vivo* bioluminescence imaging of the abdominal cavity and main organs collected from different treatment groups. **FIG. 6D** shows Hematoxylin and eosin (H&E) staining of ovarian sections in different treatment groups. **FIG. 6E** shows photographs of the peritoneum in different treatment groups (taken with iphone 14 Pro Max). **FIG. 6F** shows data analysis of the

normalized intensities of the bioluminescence signals of the mice after different treatments. **FIG. 6G** shows survival of ID8-Luc tumor-bearing mice. The mice are euthanized when they develop ascites and have a weight increase >30%. **FIG. 6H** shows ascites that were collected for volume measurement. **FIG. 6I** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs after different treatments. **FIG. 6J-6L** show the levels of pro-inflammatory cytokines (including M-CSF, IFN- $\gamma$ , and IL-6) in the blood. Macrophage colony-stimulating factor, M-CSF; Interferon- $\gamma$ , IFN- $\gamma$ ; Interleukin 6, IL-6. **FIGs. 6M-6O** show the levels of pro-inflammatory chemokines (including MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ ). Monocyte chemoattractant protein-1, MCP-1; Macrophage inflammatory protein-1 $\alpha$ , MIP-1 $\alpha$ ; Macrophage inflammatory protein-1 $\beta$ , MIP-1 $\beta$ . Data are presented as mean  $\pm$  s.d. from  $n$  independent experiments (f and g,  $n = 6$ ; h, i, j, k, l, m, n, and o,  $n = 3$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test for h, i, j, k, l, m, n, and o, and two-way ANOVA with Tukey's test for f, log-rank (Mantel-Cox) test for g. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIGs. 7A-7C** show the characterization of adjuvant LNP and its LN-targeting ability. **FIG. 7A** shows the particle size of adjuvant LNPs. **FIG. 7B** shows representative IVIS images of fluorescence distribution in BALB/c mice after SC injection of DiR-labelled adjuvant LNPs at the tail base for 6 hours. **FIG. 7C** shows quantitative analysis of the bioluminescent signals in LNs. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ).

**FIGs. 8A-8F** show TAgD-TVac<sub>OVA</sub>-mediated proteasomal degradation of OVA in BMDCs. **FIG. 8A** shows flow cytometry analysis of the internalization of different formulations by DC2.4 cells through analyzing the fluorescence of GFP. **FIGs. 8B & 8C** show quantitative analysis of co-localization of GFP signals with pVHL signals. The coefficients are close to 1 if they are highly colocalized. **FIG. 8D** shows SDS polyacrylamide gel electrophoresis of OVA and pre-fused AHPC-OVA. **FIG. 8E** shows fluorescence spectra of the mixture of pre-fused AHPC-OVA and pVHL after 1-hour incubation. The mixture of OVA, AHPC, and pVHL was used as comparison. pVHL OVA, and pVHL were pre-labelled with FITC and RITC, respectively. **FIG. 8F** shows Western blotting analysis of OVA levels in the cytoplasm in BMDCs after incubating with different LNP formulations for 3 hours. The OVA levels are evaluated after further 24-hour incubation. LNP/protein = 10/1, w/w. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ). Statistical significance was analyzed by t test. (\* $p < 0.05$ )

**FIGs. 9A-9C** show TAgD-TVac<sub>OVA</sub>-mediated antigen cross-presentation in BMDCs. **FIG. 9A** shows fluorescence images of the expression of H2kb-SIINFEKL complex on BMDCs (staining with anti-CD11c antibodies). **FIGs. 9B & 9C** show flow cytometry analysis of the population of CD80<sup>+</sup>CD86<sup>+</sup> BMDCs after different treatments. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test. (\*\* $p < 0.01$ )

**FIGs. 10A-10C** show the LN-targeting ability of Cy5.5-labelled TAgD-TVac<sub>OVA</sub> in C57BL/6 mice. **FIG. 10A** shows representative IVIS images of fluorescence distribution in C57BL/6 mice after SC injection of Cy5.5-labelled TAgD-TVac<sub>OVA</sub> for 6 hours. Pre-fused AHPC-OVA is pre-labelled with Cy5.5. **FIG. 10B** shows that TAgD-TVac<sub>OVA</sub> reaches the LNs via afferent lymphatic vessels. **FIG. 10C** shows *ex vivo* fluorescence images of the main organs.

**FIG. 11** shows delivery of GFP-Cre to LNs in Ai14 mice using adjuvant LNP. Percentage of tdTomato-positive (tdTomato<sup>+</sup>) cells in different types of immunocytes after SC injection of GFP-Cre-loaded adjuvant LNPs at tail base for 48 hours. Naked GFP-Cre is used as a control.

**FIGs. 12A & 12B** show the anti-tumor effects of TAgD-TVac<sub>OVA</sub> in a melanoma mouse model. **FIG. 12A** photographs of B16F10-OVA tumors in different treatment groups. **FIG. 12B** shows hematoxylin and eosin (H&E) staining of the tumor tissues.

**FIG. 13A** shows gating strategy for identification of CD11C<sup>+</sup> cells within the lymph nodes. **FIG. 13B** shows gating strategy for identification of CD3<sup>+</sup> T cells within the tumor tissues. **FIG. 13C** shows gating strategy for identification of CD3<sup>+</sup>CD8<sup>+</sup> T cells in the spleens.

**FIGs. 14A-14H** show anti-tumor immunity induced by TAgD-TVac<sub>OVA</sub> in a melanoma mouse model. **FIG. 14A** shows flow cytometry analysis of the expression of H2kb-SIINFEKL in DCs (gated on CD11C<sup>+</sup> cells) within LNs after different treatments. **FIGs. 4B & 14F** show flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs. **FIG. 14C** shows flow cytometry analysis of the population of CD8<sup>+</sup> T cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 14D** shows flow cytometry analysis of the expression of GzmB<sup>high</sup> in CD8<sup>+</sup> T cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIGs. 14E & 14G** Flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> and CD62L<sup>high</sup>CD44<sup>high</sup> T cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. **FIG. 14H** shows average tumor growth kinetics in CD8<sup>+</sup> T-cell-depleted mice. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments (f and g,  $n = 3$ ; h,  $n = 5$ ). Statistical significance was analyzed by one-

way ANOVA with Tukey's multiple comparisons test for f and g, and two-way ANOVA with Tukey's test for h. (\*\* $p < 0.001$ , \*\*\* $p < 0.0001$ ).

**FIG. 15A & 15B** show the pro-inflammatory cytokines and chemokines in the blood in a melanoma mouse model. **FIG. 15A** shows the levels of pro-inflammatory cytokines (including M-CSF, IFN- $\gamma$ , and IL-6) after different treatments. Macrophage colony-stimulating factor, M-CSF; Interferon- $\gamma$ , IFN- $\gamma$ ; Interleukin 6, IL-6. **FIG. 15B** shows the levels of pro-inflammatory chemokines (including MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ ). Monocyte chemoattractant protein-1, MCP-1; Macrophage inflammatory protein-1 $\alpha$ , MIP-1 $\alpha$ ; Macrophage inflammatory protein-1 $\beta$ , MIP-1 $\beta$ . Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIG. 16** shows the anti-tumor effects of TAgD-TVac<sub>4T1</sub> in a breast cancer mouse model. H&E staining of the tumor tissues and lungs.

**FIGs. 17A-17E** show Anti-tumor immunity induced by TAgD-TVac<sub>4T1</sub> in a breast cancer mouse model. **FIG. 17A** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs. **FIG. 17B** shows flow cytometry analysis of the population of CD8<sup>+</sup> T cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIG. 17C** shows flow cytometry analysis of the expression of GzmB<sup>high</sup> in CD8<sup>+</sup> T cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in tumor tissues. **FIGs. 17D & 17E** show flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> and CD62L<sup>high</sup>CD44<sup>high</sup> T cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ). Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test. (\* $p < 0.05$ , \*\*\* $p < 0.001$ ).

**FIG. 18** shows the anti-tumor effects of TAgD-TVac<sub>LLC</sub> in an orthotopic lung cancer mouse model. H&E staining of the lungs.

**FIG.s 19A-19E** show the anti-tumor immunity induced by TAgD-TVac<sub>LLC</sub> in an orthotopic lung cancer mouse model. **FIG. 19A** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs. **FIG. 19B & 19C** show flow cytometry analysis of the CD62L<sup>low</sup>CD44<sup>high</sup> and CD62L<sup>high</sup>CD44<sup>high</sup> T cells (gated on CD3<sup>+</sup>CD8<sup>+</sup> cells) in the spleens. **FIG. 19D & 19E** show flow cytometry analysis of the population of CD8<sup>+</sup> T cells (gated on CD45<sup>+</sup>CD3<sup>+</sup> cells) in LLC-Luc tumor tissues. Data are presented as mean  $\pm$  standard deviation (s.d.) from  $n$  independent experiments ( $n = 3$ ).

Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test. (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

**FIGs. 20A-20C** show the anti-tumor effects of TAgD-TVac<sub>ID-8</sub> in an inoperable ovarian cancer model. **FIGs. 20A & 20B** show H&E staining of the ovaries and spleens. T, tumor; F, follicle; CL, corpora lutea. **FIG. 20C** shows flow cytometry analysis of the expression of CD80 and CD86 in DCs (gated on CD11C<sup>+</sup> cells) within LNs.

### **DETAILED DESCRIPTION OF THE INVENTION**

Generating robust antigen-specific T-cell based cellular immunity capable of targeting and clearing tumor cells is desired for development of effective tumor vaccines. Cytotoxic T-cells initiate immune responses via recognizing peptide-major histocompatibility complex class I (MHC-I) complexes on the surface of antigen presenting cells (APCs). Tumor antigen processing and MHC-I cross-presentation are crucial steps. Specifically, antigens in tumor vaccines must be 1) endocytosed by APCs, 2) trafficked to the cytoplasm from endo-lysosomes, and 3) degraded into short peptides (approximately 8–13 amino acids) via the ubiquitin-proteasome pathway (UPP). These peptide fragments are subsequently loaded onto MHC-I binding grooves and located to the APC surface for cross-priming. Strategies using cell-penetrating peptides<sup>4</sup>, 'proton sponge' polymers, and pore-formation molecules have been developed to facilitate endo-lysosomal escape of tumor antigens to boost their immunogenicity. However, development of highly efficient anti-tumor vaccines is still a challenge due to limited antigen presentation efficiency. Improving endo-lysosomal escape can increase the cytosolic amount of tumor antigens to amplify MHC-I-dependent cross-presentation. This also increases the risk of digestive enzymes leaking from endo-lysosomes, which cause cell necrosis. Thus, maximizing the conversion of escaped tumor antigens into antigenic peptides is expected to improve anti-tumor activity, but is seldom investigated.

In UPP-mediated protein degradation, a rate-limiting step is the recruitment of E3 ubiquitin ligases to a protein of interest (POI). However, degradation of tumor antigens to antigenic peptides via UPP is actually not a high-efficient process due to the lack of a mechanism for the escaped tumor antigens to bind to E3 ubiquitin ligases. Additionally, the macromolecular crowding effect within living cells would further reduce the probability of binding between tumor antigens and E3 ubiquitin ligases.

Recent advances in chemical biology developed a powerful tool called proteolysis targeting chimera (PROTAC), which can target specific proteins for ubiquitination and proteasomal degradation by recruiting E3 ubiquitin ligases to a POI. Inspired by these

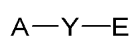
discoveries, we herein demonstrated a targeted antigen degradation-based tumor vaccine (TAgD-TVac) using tumor antigens pre-fused with PROTACs, which targeted tumor antigens to the UPP for amplified cross-presentation and robust cytotoxic T-cell activation. TAgD-TVac was constructed by loading pre-fused PROTAC tumor antigens into an adjuvant lipid nanoparticle (LNP) with superior lymph node (LN)-targeting capability. An E3 ubiquitin ligase ligands was coupled tumor antigens to obtain pre-fused PROTAC tumor antigens. LNs are the primary organs that perform immune surveillance and activation, and contain various lymphocytes including T-cells, B cells, and APCs. TAgD-TVac efficiently accumulated in LNs after subcutaneous (SC) administration (**FIG. 1**). Mechanistic research showed that adjuvant LNPs might be first taken up by APCs, including dendritic cells (DCs) and macrophages, then reached LNs through lymphocyte homing. In APCs, pre-fused PROTAC tumor antigens were released from LNPs and bound to E3 ubiquitin ligases under the guidance of E3 ligase ligands, thereby undergoing ubiquitination and proteasome-mediated antigen degradation. The resulting antigenic peptides efficiently bound to MHC-I, inducing robust cytotoxic T-cell cross-priming (**FIG. 1**). Amplified cross-presentation was confirmed by formulating TAgD-TVac with ovalbumin (OVA, as a model antigen). We found that the APC treated with TAgD-TVac exhibited stronger cross-presentation of OVA-derived peptides to MHC-I on its surface. As a result, TAgD-TVac triggered enhanced cross-priming of cytotoxic T-cells and robust anti-tumor immunity in an OVA-expressing melanoma murine model.

To assess the generality of TAgD-TVac and its clinical translatability, we utilized TAgD-TVacs to combat multiple types of solid tumors, including triple-negative breast cancer (subcutaneous tumor model), Lewis lung carcinoma (orthotopic tumor model), and ovarian cancer (clinically inoperable tumor model) (**FIG. 1**). Tumor lysate proteins (TLPs) isolated from tumor cells were used for the preparation of personalized TAgD-TVacs. We found that the TAgD-TVacs significantly inhibited tumor growth, metastasis, and recurrence. Furthermore, TAgD-TVacs could work synergistically with immune checkpoint blockade (ICB) therapy to further improve the therapeutic outcomes, paving a promising way for potentially increasing the objective response rates clinically.

PROTVAC delivers the pre-fused PROTAC tumor antigens into APCs and then to the UPP under the guidance of E3 ligase ligands, which amplifies MHC-I pathway-mediated antigen cross-presentation and initiates cytotoxic T cell activation within LNs. Compared to conventional approaches to enhance cytosolic transport of tumor antigens, our PROTVAC strategy does not require deliberate disruption of endo-lysosomes, making it an attractive solution to boost tumor antigen immunogenicity and trigger adaptive anti-tumor immunity.

Disclosed herein are exemplary personalized PROTVAC tumor vaccines using relevant tumor lysates, and successfully demonstrated their ability to elicit potent antigen-specific adaptive immunity and immunological memory against multiple solid tumors. Combining PROTVAC with ICB therapy further inhibited tumor growth and reduced the risk of tumor metastasis and recurrence, which holds translational potential. The efforts provide a novel route to initiate an adaptive immune response via simple chemistry and nanotechnology, informing the design of effective cancer immunotherapies. The success in PROTAC-based vaccine technology may also represent an important breakthrough at the interface between basic research of ubiquitin–proteasome proteolytic systems and vaccine development.

In one aspect, the present disclosure provides compounds having a structure represented by formula I or a pharmaceutically acceptable salt thereof:



I

wherein,

A is a tumor antigen;

E is an active agent (e.g., a drug); and

Y is a linker which covalently links A to E and comprises an alkylene, alkenylene,

alkynylene, cycloalkylene, heterocyclylene, arylene, or heteroarylene, or a

combination thereof, wherein the linker comprises 1-100 carbon atoms, preferably 10-50 carbon atoms.

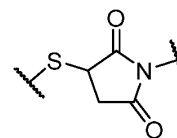
In certain embodiments, the linker is heteroalkylene comprising 5-50 moieties selected from  $-CH_2-$ ,  $-C(=O)-$ ,  $-C(=O)-NR^1-$ ,  $-NR^1-C(=O)-$ ,  $-C(=O)-O-$ ,  $-O-C(=O)-$ ,  $-NR^1-C(=O)-NR^2-$ ,  $-O-C(=O)-NR^1-$ ,  $-NR^1-C(=O)-O-$ ,  $-O-$ , and  $-S-$ ; wherein each  $R^1$  and  $R^2$  is independently selected from H and alkyl. In certain embodiments, the linker is a heteroalkylene comprising 5-50 moieties selected from  $-CH_2-$  and  $-O-$ .

In certain preferred embodiments, the linker comprises ethylene glycol. In certain further preferred embodiments, the linker comprises 1-10 ethylene glycol moieties (e.g., 2, 4, 6, or 8 ethylene glycol moieties).

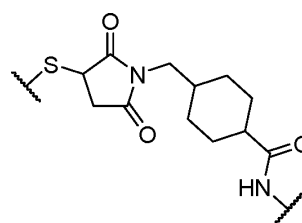
In certain embodiments, the linker is an alkylene comprising 5-50 methylene moieties.

In certain embodiments, the linker further comprises a moiety selected from  $-C(=O)-$ ,  $-C(=O)-NR^2-$ ,  $-NR^2-C(=O)-$ ,  $-C(=O)-O-$ ,  $-O-C(=O)-$ ,  $-NR^2-C(=O)-NR^3-$ ,  $-O-C(=O)-NR^2-$ ,  $-NR^2-C(=O)-O-$ ,  $-O-$ , and  $-S-$ ; wherein each  $R^2$  and  $R^3$  is independently selected from H and alkyl.

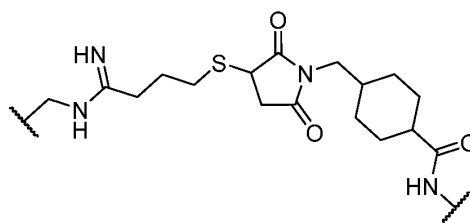
In certain embodiments, the linker further comprises 1-5  $-C(=O)-$ ,  $-C(=O)-NR^2-$  or  $-NR^2-C(=O)-$  moieties (e.g., 1, 2, or 3 moieties); wherein each  $R^2$  and  $R^3$  is independently selected from H and alkyl. In certain preferred embodiments, at least one  $-C(=O)-$  moiety is adjacent to E.



In certain embodiments, the linker further comprises . In certain

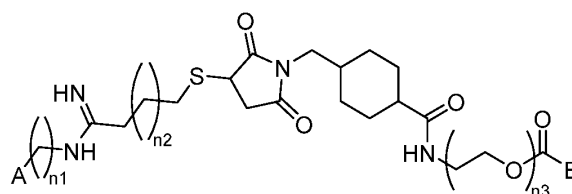


embodiments, the linker further comprises . In certain



embodiments, the linker further comprises .

In certain embodiments the compound has a structure represented by formula Ia or a pharmaceutically acceptable salt thereof:



Ia

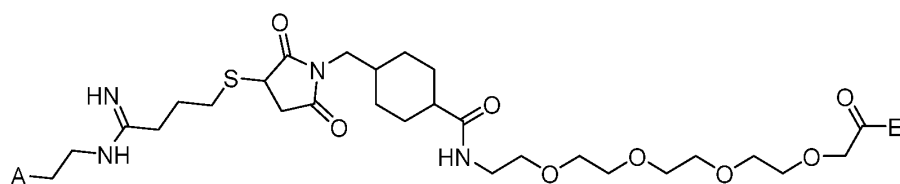
wherein

$n_1$  and  $n_2$  are each independently selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,

16, 17, 18, 19, or 20; and

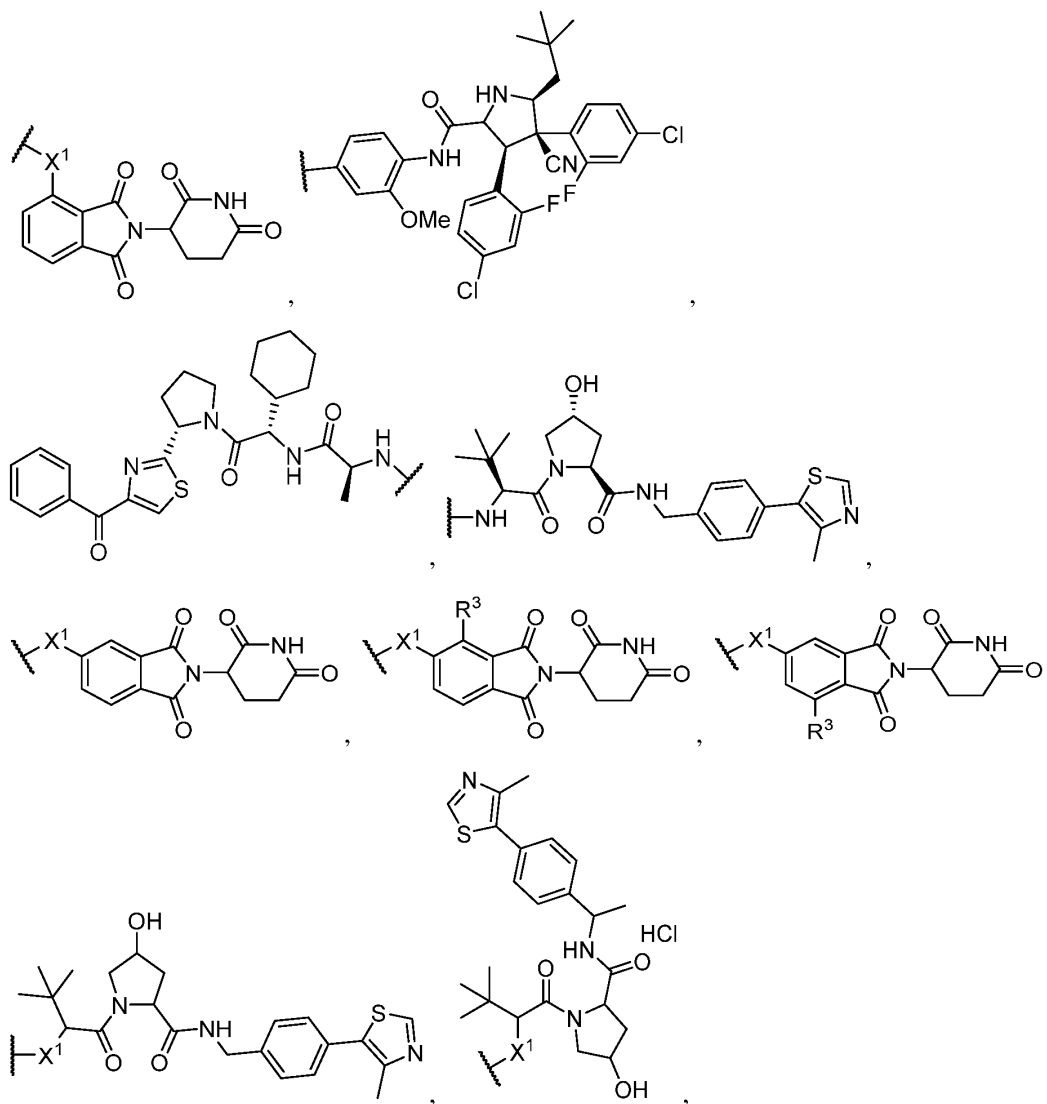
$n_3$  is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

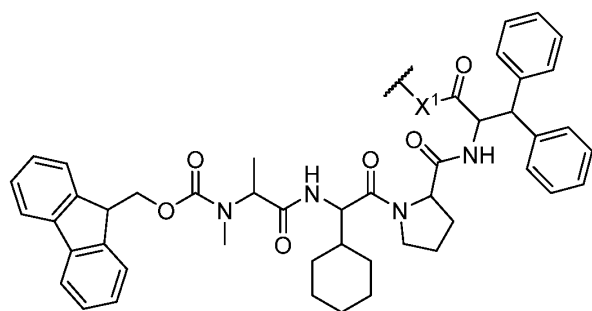
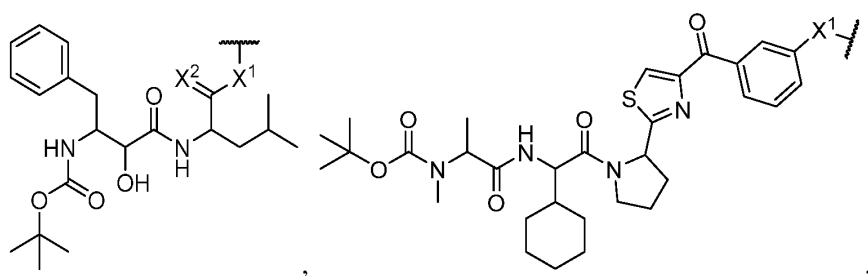
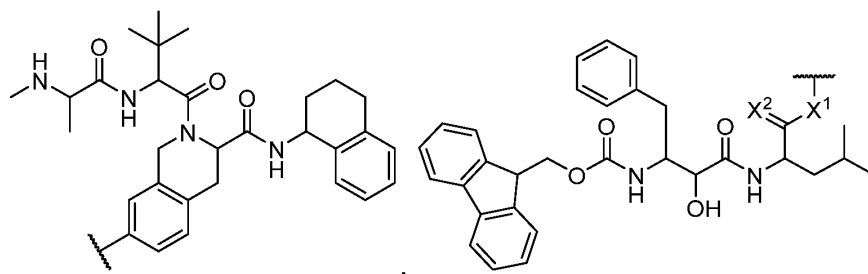
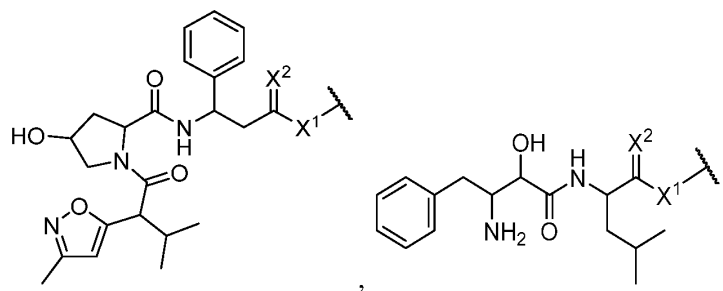
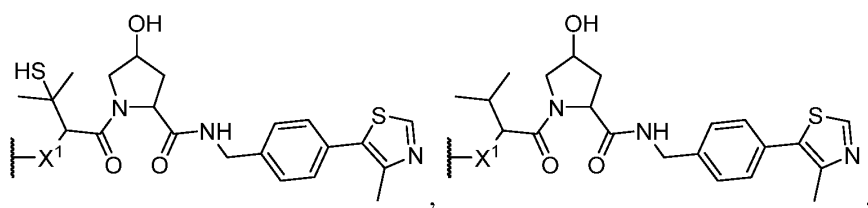
In certain embodiments, the compound has a structure represented by formula Ib or a pharmaceutically acceptable salt thereof:

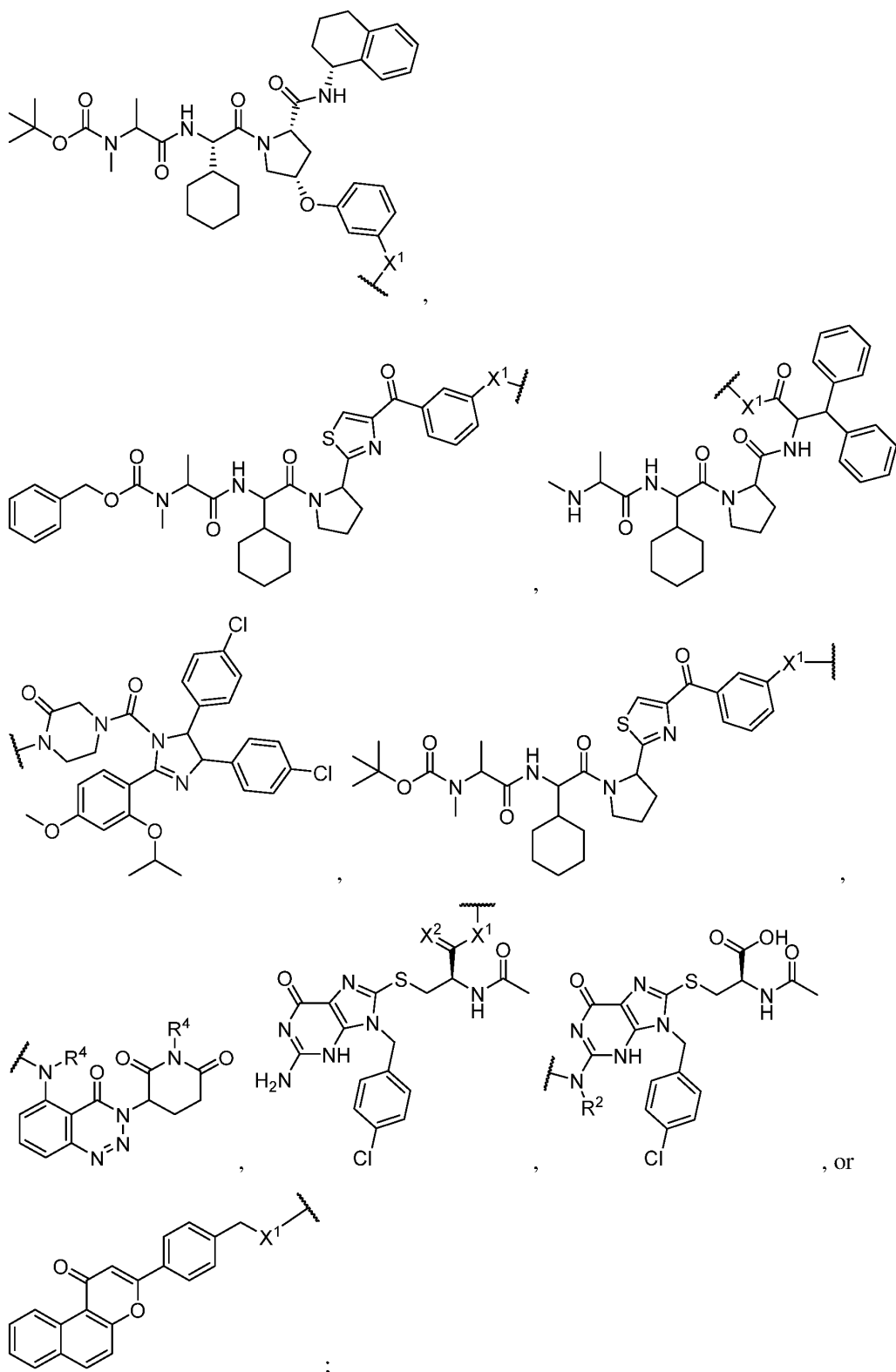


Ib.

In certain embodiments, E is a ligand for a ligase (*e.g.*, an E3 ligase). In certain embodiments, E is a ligand for a von Hippel-Lindau (VHL), cellular inhibitor of apoptosis (cIAP), cereblon (CRBN), or murine double minute 2 (MDM2). In certain embodiments, E is

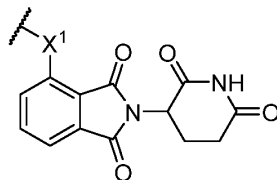






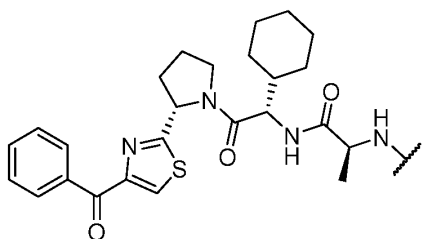
each  $X^1$  and  $X^2$  is independently selected from  $O$ ,  $NR^4$ , and  $S$ ;

each  $R^3$  is independently selected from halo (e.g., fluoro or bromo); and  
each  $R^4$  is independently H, alkyl, or aralkyl.

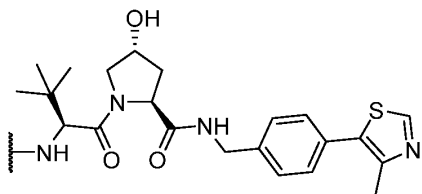


In certain embodiments, E is

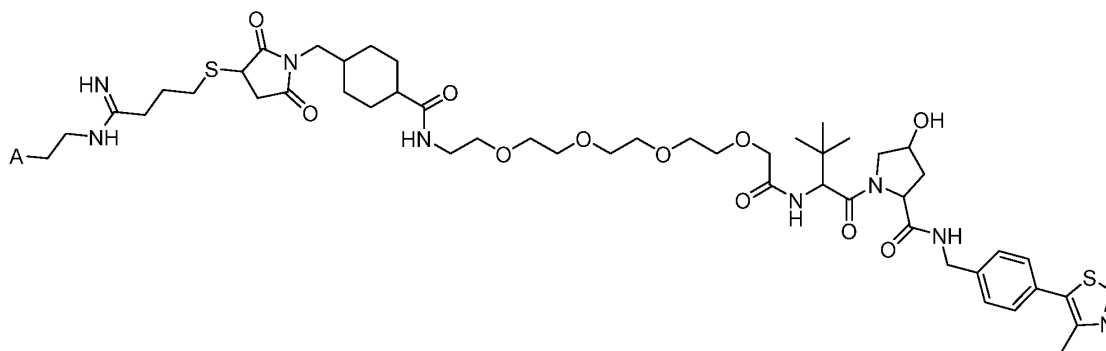
. In other embodiments, E is



. In yet other embodiments, E is

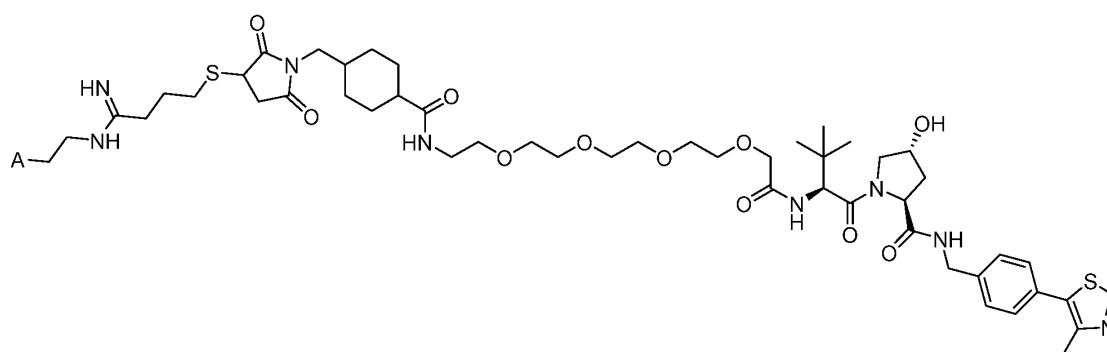


In certain embodiments, wherein the compound has a structure represented by formula Ic or a pharmaceutically acceptable salt thereof:



Ic.

In certain embodiments, the compound has a structure represented by formula Id or a pharmaceutically acceptable salt thereof:

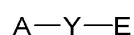


Id.

In certain embodiments, the tumor antigen is an antigenic substance produced in tumor cells that triggers an immune response in the host. In certain embodiments, A is an T antigen. In certain embodiments, the antigen is from a tumor (e.g., from tumor cells). In certain embodiments, the antigen is from an infection pathogen (e.g., a bacterium or virus). In certain embodiments, the antigen is from prostate cancer tumor cells, liver tumor cancer cells, colorectal tumor cancer cells, ovarian tumor cancer cells, pancreatic tumor cancer cells, ovarian tumor cancer cells, breast tumor cancer cells, melanoma tumor cancer cells, or bladder tumor cancer cells. In certain embodiments, the antigen is prostate antigen (PSA), alphafetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA-125), mucin-1 (MUC-1), melanoma-associated antigen (MAGE), or New York esophageal squamous cell carcinoma 1 (NY-ESO-1).

In another aspect, the present disclosure provides nanoparticles comprising a tumor antigen covalently linked to an active agent; and a plurality of lipids or lipidoids.

In certain embodiments, the tumor antigen covalently linked to an active agent has a structure represented by formula I:



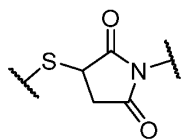
I

A is a tumor antigen;

E is an active agent (e.g., a drug); and

Y is a linker which covalently links A to E and comprises an alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene, arylene, or heteroarylene, or a combination thereof, wherein L comprises 1-100 carbon atoms, preferably 10-50 carbon atoms.

In certain embodiments, the tumor antigen is covalently linked to the active agent via



In certain embodiments, the active agent is a compound disclosed herein.

In certain embodiments, the nanoparticle comprises a plurality of lipidoids. In certain embodiments, the nanoparticle comprises a plurality of lipids. In certain preferred embodiments, the nanoparticle comprises a plurality of lipids and lipidoids. In certain embodiments, the lipids and lipidoids are selected from the group consisting of 113-O12B, cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG<sub>2k</sub>).

In another aspect, the present disclosure provides pharmaceutical compositions comprising a nanoparticle disclosed herein; and a pharmaceutically acceptable excipient.

In another aspect, the present disclosure provides methods of treating cancer in a subject in need thereof comprising administering a therapeutically effective amount of the compounds or nanoparticles disclosed herein. In certain embodiments, the cancer is melanoma, breast cancer, ovarian cancer, spleen cancer, or lung cancer. In certain embodiments, the method further comprises administering an immune checkpoint inhibitor.

In another aspect, the present disclosure provides methods of treating an infectious disease in a subject in need thereof comprising administering a therapeutically effective amount of the compounds or nanoparticles disclosed herein. In certain embodiments, the infectious disease is a bacteria. In certain embodiments, the infectious disease is a virus.

#### Pharmaceutical Compositions

The compositions and methods of the present invention may be utilized to treat an individual in need thereof. In certain embodiments, the individual is a mammal such as a human, or a non-human mammal. When administered to an animal, such as a human, the composition or the compound is preferably administered as a pharmaceutical composition comprising, for example, a compound of the invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil, or injectable organic esters. In preferred embodiments, when such pharmaceutical compositions are for human administration, particularly for invasive routes of administration (i.e., routes, such as injection or implantation,

that circumvent transport or diffusion through an epithelial barrier), the aqueous solution is pyrogen-free, or substantially pyrogen-free. The excipients can be chosen, for example, to effect delayed release of an agent or to selectively target one or more cells, tissues or organs. The pharmaceutical composition can be in dosage unit form such as tablet, capsule (including sprinkle capsule and gelatin capsule), granule, lyophile for reconstitution, powder, solution, syrup, suppository, injection or the like. The composition can also be present in a transdermal delivery system, e.g., a skin patch. The composition can also be present in a solution suitable for topical administration, such as a lotion, cream, or ointment.

A pharmaceutically acceptable carrier can contain physiologically acceptable agents that act, for example, to stabilize, increase solubility or to increase the absorption of a compound such as a compound of the invention. Such physiologically acceptable agents include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. The choice of a pharmaceutically acceptable carrier, including a physiologically acceptable agent, depends, for example, on the route of administration of the composition. The preparation or pharmaceutical composition can be a selfemulsifying drug delivery system or a selfmicroemulsifying drug delivery system. The pharmaceutical composition (preparation) also can be a liposome or other polymer matrix, which can have incorporated therein, for example, a compound of the invention. Liposomes, for example, which comprise phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

A pharmaceutical composition (preparation) can be administered to a subject by any of a number of routes of administration including, for example, orally (for example, drenches as in aqueous or non-aqueous solutions or suspensions, tablets, capsules (including sprinkle capsules and gelatin capsules), boluses, powders, granules, pastes for application to the tongue); absorption through the oral mucosa (e.g., sublingually); subcutaneously; transdermally (for example as a patch applied to the skin); and topically (for example, as a cream, ointment or spray applied to the skin). The compound may also be formulated for inhalation. In certain embodiments, a compound may be simply dissolved or suspended in sterile water. Details of appropriate routes of administration and compositions suitable for same can be found in, for example, U.S. Pat. Nos. 6,110,973, 5,763,493, 5,731,000, 5,541,231, 5,427,798, 5,358,970 and 4,172,896, as well as in patents cited therein.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient

which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

Methods of preparing these formulations or compositions include the step of bringing into association an active compound, such as a compound of the invention, with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules (including sprinkle capsules and gelatin capsules), cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), lyophile, powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. Compositions or compounds may also be administered as a bolus, electuary or paste.

To prepare solid dosage forms for oral administration (capsules (including sprinkle capsules and gelatin capsules), tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and

mixtures thereof; (10) complexing agents, such as, modified and unmodified cyclodextrins; and (11) coloring agents. In the case of capsules (including sprinkle capsules and gelatin capsules), tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions, such as dragees, capsules (including sprinkle capsules and gelatin capsules), pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions that can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms useful for oral administration include pharmaceutically acceptable emulsions, lyophiles for reconstitution, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, cyclodextrins and derivatives thereof, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame

oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an active compound, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the active compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the compound in a polymer matrix or gel.

Pharmaceutical compositions suitable for parenteral administration comprise one or more active compounds in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the

formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsulated matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissue.

For use in the methods of this invention, active compounds can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals.

A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a compound at a particular target site.

Actual dosage levels of the active ingredients in the pharmaceutical compositions may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound or combination of compounds employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound(s) being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound(s) employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the therapeutically effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the pharmaceutical composition or compound at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. By “therapeutically effective amount” is meant the concentration of a compound that is sufficient to elicit the desired therapeutic effect. It is generally understood that the effective amount of the compound will vary according to the weight, sex, age, and medical history of the subject. Other factors which influence the effective amount may include, but are not limited to, the severity of the patient's condition, the disorder being treated, the stability of the compound, and, if desired, another type of therapeutic agent being administered with the compound of the invention. A larger total dose can be delivered by multiple administrations of the agent. Methods to determine efficacy and dosage are known to those skilled in the art (Isselbacher et al. (1996) Harrison's Principles of Internal Medicine 13 ed., 1814-1882, herein incorporated by reference).

In general, a suitable daily dose of an active compound used in the compositions and methods of the invention will be that amount of the compound that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

If desired, the effective daily dose of the active compound may be administered as one, two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. In certain embodiments of the present invention, the active compound may be administered two or three times daily. In preferred embodiments, the active compound will be administered once daily.

The patient receiving this treatment is any animal in need, including primates, in particular humans; and other mammals such as equines, cattle, swine, sheep, cats, and dogs; poultry; and pets in general.

In certain embodiments, compounds of the invention may be used alone or conjointly administered with another type of therapeutic agent.

The present disclosure includes the use of pharmaceutically acceptable salts of compounds of the invention in the compositions and methods of the present invention. In certain embodiments, contemplated salts of the invention include, but are not limited to, alkyl, dialkyl, trialkyl or tetra-alkyl ammonium salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, L-arginine, benenthamine, benzathine, betaine, calcium hydroxide, choline, deanol, diethanolamine, diethylamine, 2-(diethylamino)ethanol, ethanolamine, ethylenediamine, N-methylglucamine, hydrabamine, 1H-imidazole, lithium, L-lysine, magnesium, 4-(2-hydroxyethyl)morpholine, piperazine, potassium, 1-(2-hydroxyethyl)pyrrolidine, sodium, triethanolamine, tromethamine, and zinc salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, Na, Ca, K, Mg, Zn or other metal salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, 1-hydroxy-2-naphthoic acid, 2,2-dichloroacetic acid, 2-hydroxyethanesulfonic acid, 2-oxoglutaric acid, 4-acetamidobenzoic acid, 4-aminosalicylic acid, acetic acid, adipic acid, l-ascorbic acid, l-aspartic acid, benzenesulfonic acid, benzoic acid, (+)-camphoric acid, (+)-camphor-10-sulfonic acid, capric acid (decanoic acid), caproic acid (hexanoic acid), caprylic acid (octanoic acid), carbonic acid, cinnamic acid, citric acid, cyclamic acid, dodecylsulfuric acid, ethane-1,2-disulfonic acid, ethanesulfonic acid, formic acid, fumaric acid, galactaric acid, gentisic acid, d-glucoheptonic acid, d-gluconic acid, d-glucuronic acid, glutamic acid, glutaric acid, glycerophosphoric acid, glycolic acid, hippuric acid, hydrobromic acid, hydrochloric acid, isobutyric acid, lactic acid, lactobionic acid, lauric acid, maleic acid, l-malic acid, malonic acid, mandelic acid, methanesulfonic acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, nicotinic acid, nitric acid, oleic acid, oxalic acid, palmitic acid, pamoic acid, phosphoric acid, propionic acid, l-pyrogutamic

acid, salicylic acid, sebacic acid, stearic acid, succinic acid, sulfuric acid, l-tartaric acid, thiocyanic acid, p-toluenesulfonic acid, trifluoroacetic acid, and undecylenic acid acid salts.

The pharmaceutically acceptable acid addition salts can also exist as various solvates, such as with water, methanol, ethanol, dimethylformamide, and the like. Mixtures of such solvates can also be prepared. The source of such solvate can be from the solvent of crystallization, inherent in the solvent of preparation or crystallization, or adventitious to such solvent.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: (1) water-soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal-chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

#### Definitions

Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, chemistry, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology, immunology, microbiology, pharmacology, genetics and protein and nucleic acid chemistry, described herein, are those well known and commonly used in the art.

The methods and techniques of the present disclosure are generally performed, unless otherwise indicated, according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout this specification. See, e.g. "Principles of Neural Science", McGraw-Hill Medical, New York, N.Y. (2000); Motulsky, "Intuitive Biostatistics", Oxford University Press, Inc. (1995); Lodish et al., "Molecular Cell Biology, 4th ed.", W. H. Freeman & Co., New York (2000); Griffiths et al., "Introduction to Genetic Analysis, 7th ed.", W. H. Freeman & Co., N.Y. (1999); and Gilbert et al., "Developmental Biology, 6th ed.", Sinauer Associates, Inc., Sunderland, MA (2000).

Chemistry terms used herein, unless otherwise defined herein, are used according to conventional usage in the art, as exemplified by “The McGraw-Hill Dictionary of Chemical Terms”, Parker S., Ed., McGraw-Hill, San Francisco, C.A. (1985).

All of the above, and any other publications, patents and published patent applications referred to in this application are specifically incorporated by reference herein. In case of conflict, the present specification, including its specific definitions, will control.

The term “agent” is used herein to denote a chemical compound (such as an organic or inorganic compound, a mixture of chemical compounds), a biological macromolecule (such as a nucleic acid, an antibody, including parts thereof as well as humanized, chimeric and human antibodies and monoclonal antibodies, a protein or portion thereof, e.g., a peptide, a lipid, a carbohydrate), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents include, for example, agents whose structure is known, and those whose structure is not known.

A “patient,” “subject,” or “individual” are used interchangeably and refer to either a human or a non-human animal. These terms include mammals, such as humans, primates, livestock animals (including bovines, porcines, etc.), companion animals (e.g., canines, felines, etc.) and rodents (e.g., mice and rats).

“Treating” a condition or patient refers to taking steps to obtain beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term “preventing” is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount.

“Administering” or “administration of” a substance, a compound or an agent to a subject can be carried out using one of a variety of methods known to those skilled in the art. For example, a compound or an agent can be administered, intravenously, arterially, intradermally, intramuscularly, intraperitoneally, subcutaneously, ocularly, sublingually, orally (by ingestion), intranasally (by inhalation), intraspinally, intracerebrally, and transdermally (by absorption, e.g., through a skin duct). A compound or agent can also appropriately be introduced by rechargeable or biodegradable polymeric devices or other devices, e.g., patches and pumps, or formulations, which provide for the extended, slow or controlled release of the compound or agent. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

Appropriate methods of administering a substance, a compound or an agent to a subject will also depend, for example, on the age and/or the physical condition of the subject and the chemical and biological properties of the compound or agent (e.g., solubility, digestibility, bioavailability, stability and toxicity). In some embodiments, a compound or an agent is administered orally, e.g., to a subject by ingestion. In some embodiments, the orally administered compound or agent is in an extended release or slow release formulation, or administered using a device for such slow or extended release.

As used herein, the phrase “conjoint administration” refers to any form of administration of two or more different therapeutic agents such that the second agent is administered while the previously administered therapeutic agent is still effective in the body (e.g., the two agents are simultaneously effective in the patient, which may include synergistic effects of the two agents). For example, the different therapeutic compounds can be administered either in the same formulation or in separate formulations, either concomitantly or sequentially. Thus, an individual who receives such treatment can benefit from a combined effect of different therapeutic agents.

A “therapeutically effective amount” or a “therapeutically effective dose” of a drug or agent is an amount of a drug or an agent that, when administered to a subject will have the intended therapeutic effect. The full therapeutic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a therapeutically effective amount may be administered in one or more administrations. The precise effective amount needed for a subject will depend upon, for example, the subject’s size, health and age, and the nature and extent of the condition being treated. The skilled worker can readily determine the effective amount for a given situation by routine experimentation.

As used herein, the terms “optional” or “optionally” mean that the subsequently described event or circumstance may occur or may not occur, and that the description includes instances where the event or circumstance occurs as well as instances in which it does not. For example, “optionally substituted alkyl” refers to the alkyl may be substituted as well as where the alkyl is not substituted.

It is understood that substituents and substitution patterns on the compounds of the present invention can be selected by one of ordinary skilled person in the art to result chemically stable compounds which can be readily synthesized by techniques known in the art, as well as those methods set forth below, from readily available starting materials. If a substituent is itself substituted with more than one group, it is understood that these multiple groups may be on the same carbon or on different carbons, so long as a stable structure results.

As used herein, the term “optionally substituted” refers to the replacement of one to six hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: hydroxyl, hydroxyalkyl, alkoxy, halogen, alkyl, nitro, silyl, acyl, acyloxy, aryl, cycloalkyl, heterocyclyl, amino, aminoalkyl, cyano, haloalkyl, haloalkoxy, -OCO-CH<sub>2</sub>-O-alkyl, -OP(O)(O-alkyl)<sub>2</sub> or -CH<sub>2</sub>-OP(O)(O-alkyl)<sub>2</sub>. Preferably, “optionally substituted” refers to the replacement of one to four hydrogen radicals in a given structure with the substituents mentioned above. More preferably, one to three hydrogen radicals are replaced by the substituents as mentioned above. It is understood that the substituent can be further substituted.

As used herein, the term “alkyl” refers to saturated aliphatic groups, including but not limited to C<sub>1</sub>-C<sub>10</sub> straight-chain alkyl groups or C<sub>1</sub>-C<sub>10</sub> branched-chain alkyl groups. Preferably, the “alkyl” group refers to C<sub>1</sub>-C<sub>6</sub> straight-chain alkyl groups or C<sub>1</sub>-C<sub>6</sub> branched-chain alkyl groups. Most preferably, the “alkyl” group refers to C<sub>1</sub>-C<sub>4</sub> straight-chain alkyl groups or C<sub>1</sub>-C<sub>4</sub> branched-chain alkyl groups. Examples of “alkyl” include, but are not limited to, methyl, ethyl, 1-propyl, 2-propyl, n-butyl, sec-butyl, tert-butyl, 1-pentyl, 2-pentyl, 3-pentyl, neo-pentyl, 1-hexyl, 2-hexyl, 3-hexyl, 1-heptyl, 2-heptyl, 3-heptyl, 4-heptyl, 1-octyl, 2-octyl, 3-octyl or 4-octyl and the like. The “alkyl” group may be optionally substituted.

The term “acyl” is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)-, preferably alkylC(O)-.

The term “acylamino” is art-recognized and refers to an amino group substituted with an acyl group and may be represented, for example, by the formula hydrocarbylC(O)NH-.

The term “acyloxy” is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)O-, preferably alkylC(O)O-.

The term “alkoxy” refers to an alkyl group having an oxygen attached thereto. Representative alkoxy groups include methoxy, ethoxy, propoxy, tert-butoxy and the like.

The term “alkoxyalkyl” refers to an alkyl group substituted with an alkoxy group and may be represented by the general formula alkyl-O-alkyl.

The term “alkyl” refers to saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C<sub>1-30</sub> for straight chains, C<sub>3-30</sub> for branched chains), and more preferably 20 or fewer.

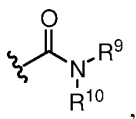
Moreover, the term “alkyl” as used throughout the specification, examples, and claims is intended to include both unsubstituted and substituted alkyl groups, the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone, including haloalkyl groups such as trifluoromethyl and 2,2,2-trifluoroethyl, etc.

The term “C<sub>x-y</sub>” or “C<sub>x</sub>-C<sub>y</sub>”, when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups that contain from x to y carbons in the chain. C<sub>0</sub>alkyl indicates a hydrogen where the group is in a terminal position, a bond if internal. A C<sub>1-6</sub>alkyl group, for example, contains from one to six carbon atoms in the chain.

The term “alkylamino”, as used herein, refers to an amino group substituted with at least one alkyl group.

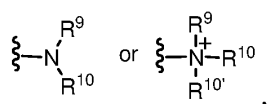
The term “alkylthio”, as used herein, refers to a thiol group substituted with an alkyl group and may be represented by the general formula alkylS-.

The term “amido”, as used herein, refers to a group



wherein R<sup>9</sup> and R<sup>10</sup> each independently represent a hydrogen or hydrocarbyl group, or R<sup>9</sup> and R<sup>10</sup> taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The terms “amine” and “amino” are art-recognized and refer to both unsubstituted and substituted amines and salts thereof, e.g., a moiety that can be represented by



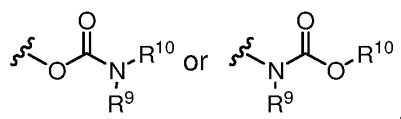
wherein  $\text{R}^9$ ,  $\text{R}^{10}$ , and  $\text{R}^{10'}$  each independently represent a hydrogen or a hydrocarbyl group, or  $\text{R}^9$  and  $\text{R}^{10}$  taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The term “aminoalkyl”, as used herein, refers to an alkyl group substituted with an amino group.

The term “aralkyl”, as used herein, refers to an alkyl group substituted with an aryl group.

The term “aryl” as used herein include substituted or unsubstituted single-ring aromatic groups in which each atom of the ring is carbon. Preferably the ring is a 5- to 7-membered ring, more preferably a 6-membered ring. The term “aryl” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Aryl groups include benzene, naphthalene, phenanthrene, phenol, aniline, and the like.

The term “carbamate” is art-recognized and refers to a group



wherein  $\text{R}^9$  and  $\text{R}^{10}$  independently represent hydrogen or a hydrocarbyl group.

The term “carbocyclalkyl”, as used herein, refers to an alkyl group substituted with a carbocycle group.

The term “carbocycle” includes 5-7 membered monocyclic and 8-12 membered bicyclic rings. Each ring of a bicyclic carbocycle may be selected from saturated, unsaturated and aromatic rings. Carbocycle includes bicyclic molecules in which one, two or three or more atoms are shared between the two rings. The term “fused carbocycle” refers to a bicyclic carbocycle in which each of the rings shares two adjacent atoms with the other ring. Each ring of a fused carbocycle may be selected from saturated, unsaturated and aromatic rings. In an exemplary embodiment, an aromatic ring, e.g., phenyl, may be fused to a saturated or unsaturated ring, e.g., cyclohexane, cyclopentane, or cyclohexene. Any combination of saturated, unsaturated and aromatic bicyclic rings, as valence permits, is included in the definition of carbocyclic. Exemplary “carbocycles” include cyclopentane, cyclohexane,

bicyclo[2.2.1]heptane, 1,5-cyclooctadiene, 1,2,3,4-tetrahydronaphthalene, bicyclo[4.2.0]oct-3-ene, naphthalene and adamantane. Exemplary fused carbocycles include decalin, naphthalene, 1,2,3,4-tetrahydronaphthalene, bicyclo[4.2.0]octane, 4,5,6,7-tetrahydro-1H-indene and bicyclo[4.1.0]hept-3-ene. “Carbocycles” may be substituted at any one or more positions capable of bearing a hydrogen atom.

The term “carbocyclalkyl”, as used herein, refers to an alkyl group substituted with a carbocycle group.

The term “carbonate” is art-recognized and refers to a group  $-\text{OCO}_2-$ .

The term “carboxy”, as used herein, refers to a group represented by the formula  $-\text{CO}_2\text{H}$ .

The term “cycloalkyl” includes substituted or unsubstituted non-aromatic single ring structures, preferably 4- to 8-membered rings, more preferably 4- to 6-membered rings. The term “cycloalkyl” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is cycloalkyl and the substituent (e.g.,  $\text{R}^{100}$ ) is attached to the cycloalkyl ring, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryl, and/or heterocyclals. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, pyrimidine, denzodioxane, tetrahydroquinoline, and the like.

The term “ester”, as used herein, refers to a group  $-\text{C}(\text{O})\text{OR}^9$  wherein  $\text{R}^9$  represents a hydrocarbyl group.

The term “ether”, as used herein, refers to a hydrocarbyl group linked through an oxygen to another hydrocarbyl group. Accordingly, an ether substituent of a hydrocarbyl group may be hydrocarbyl-O-. Ethers may be either symmetrical or unsymmetrical. Examples of ethers include, but are not limited to, heterocycle-O-heterocycle and aryl-O-heterocycle. Ethers include “alkoxyalkyl” groups, which may be represented by the general formula alkyl-O-alkyl.

The terms “halo” and “halogen” as used herein means halogen and includes chloro, fluoro, bromo, and iodo.

The terms “hetaralkyl” and “heteroaralkyl”, as used herein, refers to an alkyl group substituted with a hetaryl group.

The terms “heteroaryl” and “hetaryl” include substituted or unsubstituted aromatic single ring structures, preferably 5- to 7-membered rings, more preferably 5- to 6-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms “heteroaryl” and “hetaryl” also include

polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heteroaromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryl, and/or heterocyclyls. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like.

The term “heteroatom” as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, and sulfur.

The term “heterocyclylalkyl”, as used herein, refers to an alkyl group substituted with a heterocycle group.

The terms “heterocyclyl”, “heterocycle”, and “heterocyclic” refer to substituted or unsubstituted non-aromatic ring structures, preferably 3- to 10-membered rings, more preferably 3- to 7-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms “heterocyclyl” and “heterocyclic” also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heterocyclic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryl, and/or heterocyclyls. Heterocyclyl groups include, for example, piperidine, piperazine, pyrrolidine, morpholine, lactones, lactams, and the like.

The term “hydrocarbonyl”, as used herein, refers to a group that is bonded through a carbon atom that does not have a =O or =S substituent, and typically has at least one carbon-hydrogen bond and a primarily carbon backbone, but may optionally include heteroatoms. Thus, groups like methyl, ethoxyethyl, 2-pyridyl, and even trifluoromethyl are considered to be hydrocarbonyl for the purposes of this application, but substituents such as acetyl (which has a =O substituent on the linking carbon) and ethoxy (which is linked through oxygen, not carbon) are not. Hydrocarbonyl groups include, but are not limited to aryl, heteroaryl, carbocycle, heterocycle, alkyl, alkenyl, alkynyl, and combinations thereof.

The term “hydroxyalkyl”, as used herein, refers to an alkyl group substituted with a hydroxy group.

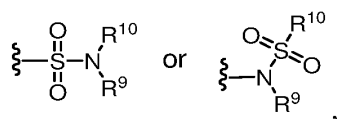
The term “lower” when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups where there are ten or fewer atoms in the substituent, preferably six or fewer. A “lower alkyl”, for example, refers to an alkyl group that contains ten or fewer carbon atoms, preferably six or fewer. In certain embodiments, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy substituents defined herein are respectively lower acyl, lower acyloxy, lower alkyl, lower alkenyl, lower alkynyl, or lower

alkoxy, whether they appear alone or in combination with other substituents, such as in the recitations hydroxyalkyl and aralkyl (in which case, for example, the atoms within the aryl group are not counted when counting the carbon atoms in the alkyl substituent).

The terms “polycyclyl”, “polycycle”, and “polycyclic” refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls) in which two or more atoms are common to two adjoining rings, e.g., the rings are “fused rings”. Each of the rings of the polycycle can be substituted or unsubstituted. In certain embodiments, each ring of the polycycle contains from 3 to 10 atoms in the ring, preferably from 5 to 7.

The term “sulfate” is art-recognized and refers to the group  $-\text{OSO}_3\text{H}$ , or a pharmaceutically acceptable salt thereof.

The term “sulfonamido” is art-recognized and refers to the group represented by the general formulae



wherein  $\text{R}^9$  and  $\text{R}^{10}$  independently represents hydrogen or hydrocarbyl.

The term “sulfoxide” is art-recognized and refers to the group  $-\text{S}(\text{O})-$ .

The term “sulfonate” is art-recognized and refers to the group  $\text{SO}_3\text{H}$ , or a pharmaceutically acceptable salt thereof.

The term “sulfone” is art-recognized and refers to the group  $-\text{S}(\text{O})_2-$ .

The term “substituted” refers to moieties having substituents replacing a hydrogen on one or more carbons of the backbone. It will be understood that “substitution” or “substituted with” includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc. As used herein, the term “substituted” is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and non-aromatic substituents of organic compounds. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. Substituents can include any substituents described herein, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an

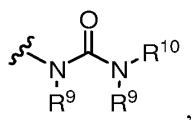
alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate.

The term “thioalkyl”, as used herein, refers to an alkyl group substituted with a thiol group.

The term “thioester”, as used herein, refers to a group  $-C(O)SR^9$  or  $-SC(O)R^9$  wherein  $R^9$  represents a hydrocarbonyl.

The term “thioether”, as used herein, is equivalent to an ether, wherein the oxygen is replaced with a sulfur.

The term “urea” is art-recognized and may be represented by the general formula



wherein  $R^9$  and  $R^{10}$  independently represent hydrogen or a hydrocarbonyl.

The term “modulate” as used herein includes the inhibition or suppression of a function or activity (such as cell proliferation) as well as the enhancement of a function or activity.

The phrase “pharmaceutically acceptable” is art-recognized. In certain embodiments, the term includes compositions, excipients, adjuvants, polymers and other materials and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase “pharmaceutically acceptable carrier” as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene

glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

“Pharmaceutically acceptable salt” or “salt” is used herein to refer to an acid addition salt or a basic addition salt which is suitable for or compatible with the treatment of patients.

The term “pharmaceutically acceptable acid addition salt” as used herein means any non-toxic organic or inorganic salt of any base compounds represented by Formula I. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulfuric and phosphoric acids, as well as metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids that form suitable salts include mono-, di-, and tricarboxylic acids such as glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, benzoic, phenylacetic, cinnamic and salicylic acids, as well as sulfonic acids such as p-toluene sulfonic and methanesulfonic acids. Either the mono or di-acid salts can be formed, and such salts may exist in either a hydrated, solvated or substantially anhydrous form. In general, the acid addition salts of compounds of Formula I are more soluble in water and various hydrophilic organic solvents, and generally demonstrate higher melting points in comparison to their free base forms. The selection of the appropriate salt will be known to one skilled in the art. Other non-pharmaceutically acceptable salts, e.g., oxalates, may be used, for example, in the isolation of compounds of Formula I for laboratory use, or for subsequent conversion to a pharmaceutically acceptable acid addition salt.

The term “pharmaceutically acceptable basic addition salt” as used herein means any non-toxic organic or inorganic base addition salt of any acid compounds represented by Formula I or any of their intermediates. Illustrative inorganic bases which form suitable salts include lithium, sodium, potassium, calcium, magnesium, or barium hydroxide. Illustrative organic bases which form suitable salts include aliphatic, alicyclic, or aromatic organic amines such as methylamine, trimethylamine and picoline or ammonia. The selection of the appropriate salt will be known to a person skilled in the art.

Many of the compounds useful in the methods and compositions of this disclosure have at least one stereogenic center in their structure. This stereogenic center may be present in a R or a S configuration, said R and S notation is used in correspondence with the rules described in Pure Appl. Chem. (1976), 45, 11-30. The disclosure contemplates all stereoisomeric forms

such as enantiomeric and diastereoisomeric forms of the compounds, salts, prodrugs or mixtures thereof (including all possible mixtures of stereoisomers). See, e.g., WO 01/062726.

Furthermore, certain compounds which contain alkenyl groups may exist as Z (zusammen) or E (entgegen) isomers. In each instance, the disclosure includes both mixture and separate individual isomers.

“Prodrug” or “pharmaceutically acceptable prodrug” refers to a compound that is metabolized, for example hydrolyzed or oxidized, in the host after administration to form the compound of the present disclosure (e.g., compounds of formula I). Typical examples of prodrugs include compounds that have biologically labile or cleavable (protecting) groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolyzed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, or dephosphorylated to produce the active compound. Examples of prodrugs using ester or phosphoramidate as biologically labile or cleavable (protecting) groups are disclosed in U.S. Patents 6,875,751, 7,585,851, and 7,964,580, the disclosures of which are incorporated herein by reference. The prodrugs of this disclosure are metabolized to produce a compound of Formula I. The present disclosure includes within its scope, prodrugs of the compounds described herein. Conventional procedures for the selection and preparation of suitable prodrugs are described, for example, in “Design of Prodrugs” Ed. H. Bundgaard, Elsevier, 1985.

The term “Log of solubility”, “LogS” or “logS” as used herein is used in the art to quantify the aqueous solubility of a compound. The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. A low solubility often goes along with a poor absorption. LogS value is a unit stripped logarithm (base 10) of the solubility measured in mol/liter.

The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

## **EXAMPLES**

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

### **Example 1: Synthesis and Activity of Exemplary PROTVACs**

#### **Characterization of pre-fused PROTAC tumor antigen and PROTVAC**

##### *The affinity between pre-fused PROTAC tumor antigen and pVHL*

TagD-TVac consists of pre-fused PROTAC tumor antigens and an adjuvant LNP with LN-targeting ability. As a proof of concept, (*S,R,S*)-AHPC, a ligand that recruits the von Hippel-Lindau protein (pVHL, an E3 ubiquitin ligase) was employed. Pre-fused PROTAC tumor antigen was synthesized by covalently modifying (*S,R,S*)-AHPC onto a tumor antigen (**FIG. 2A**). A green fluorescent protein (GFP)-Cre was first utilized (obtained by fusing Cre recombinase) as a substitute for tumor antigen to prepare the pre-fused formulation (denoted as pre-fused AHPC-GFP-Cre), due to easy visualization under excitation light. Integration of AHPC onto GFP-Cre was confirmed by assessing the affinity of pre-fused AHPC-GFP-Cre to pVHL using confocal laser scanning microscopy (CLSM). pVHL was pre-labelled with rhodamine-B-isothiocyanate (RITC), and then co-incubated with pre-fused AHPC-GFP-Cre. The mixture of GFP-Cre, AHPC, and pVHL acted as a control. As shown in **FIG. 2B**, the co-localization of pre-fused AHPC-GFP-Cre and pVHL was observed, indicating the binding between pre-fused AHPC-GFP-Cre and pVHL, and the formation of pre-fused AHPC-GFP-Cre/pVHL complexes. In contrast, negligible co-localization was observed when incubating the GFP-Cre, AHPC, and pVHL mixture. The affinity between pre-fused AHPC-GFP-Cre and pVHL was further confirmed by agarose gel electrophoresis. pVHLs were immobilized onto the surface of poly(lactic-co-glycolic acid) (PLGA) NPs, and co-incubated with pre-fused GFP-AHPC in the presence of (polyethylene glycol) PEG (**FIG. 2C**). PEG was utilized to simulate crowding conditions inside cells. Then, samples were loaded into gel wells for electrophoresis. Almost all GFP signals were observed near the gel wells when incubating pVHL-modified PLGA NPs with pre-fused AHPC-GFP-Cres (**FIG. 2D**). In contrast, due the negligible affinity between GFP-Cre and pVHL-modified PLGA NPs, all GFP-Cre ran out of the wells. These results confirmed the successful integration of AHPC onto the GFP-Cre, and demonstrated the ability of pre-fused AHPC-GFP-Cre to target pVHL.

*The ability of pre-fused PROTAC tumor antigen to target intracellular pVHL*

Then its ability to target intracellular pVHLs in mouse DCs (DC2.4) was investigated. An adjuvant LNP, with LN-targeting specificity, was constructed for intracellular delivery of pre-fused AHPC-GFP-Cre. Adjuvant LNPs were formulated with adjuvant lipidoids (113-O12B, **FIG. 2E** and **Schemes 1 & 2**), cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG<sub>2k</sub>) at a molar ratio of 48.5:38:10:3.5. Transmission electron microscopy image of adjuvant LNPs showed a closed vesicular morphology with a diameter around 120 nm (**FIGs. 2F & 7A**). This adjuvant LNP could specifically accumulate in LNs after SC injection at the tail base, which was demonstrated using DiR-labelled formulation and observed by IVIS imaging system (PerkinElmer, **FIGs. 7B & 7C**).

Next, the ability of pre-fused AHPC-GFP-Cre to recruit intracellular pVHL under the guidance of AHPC ligand was evaluated. If pre-fused AHPC-GFP-Cre cannot recruit cytosolic pVHL, GFP will be detected in nuclei due to the inherent nucleus targeting capability of Cre recombinase. Adjuvant LNPs were used to deliver pre-fused (denoted as pre-fused AHPC-GFP-Cre LNP) and co-loaded control (denoted as GFP-Cre + AHPC LNP) formulations. AHPC encapsulation was confirmed using high performance liquid chromatography (HPLC). These formulations were first incubated with DC2.4 cells for 3 hours, which was sufficient for them to be taken up (**FIG. 8A**). After an additional 9-hour incubation following cell uptake, pre-fused AHPC-GFP-Cres co-localized with pVHLs that were stained by anti-VHL antibodies, and showed poor accumulation in the nuclei as shown in **FIG. 2G**. Conversely, control GFP-Cre and AHPC showed weaker GFP-Cre and pVHL co-localization, and significant accumulation of GFP-Cre in nuclei. These results indicated that pre-fused AHPC-GFP-Cre preferentially bound intracellular pVHLs. Co-localization based on CLSM images was quantified by calculating Pearson's co-localization coefficient (*PCC*) between GFP fluorescence (green) and pVHL (red). *PCC* value in the pre-fused AHPC-GFP-Cre LNP group ( $PCC_{\text{Pre-fused AHPC-GFP-Cre LNP}} = 0.89$ ) was much higher than the GFP-Cre + AHPC LNP group ( $PCC_{\text{GFP-Cre + AHPC LNP}} = 0.58$ , **FIGs. 8B & 8C**). These results confirmed the ability of pre-fused AHPC-GFP-Cre to recruit intracellular pVHL.

**PROTVAC enhances antigen cross-presentation**

It was demonstrated that GFP-Cre fused with AHPC ligands recruited pVHLs in cell-free and in-cell levels. Next, ubiquitination and proteasome-mediated degradation of GFP-Cre in DC2.4 cells were evaluated by monitoring GFP-Cre fluorescence using flow cytometry.

Different formulations were incubated with DC2.4 cells for 3 hours, then replaced by fresh culture medium for further incubation. As shown in **FIG. 2H**, pre-fused AHPC-GFP-Cre exhibited faster GFP-Cre degradation (decreased to 8.3%) compared to GFP-Cre alone (24.7 %) and GFP-Cre + AHPC (31.3%) during 24 hours of observation. These results indicated that modification of GFP-Cre with AHPC significantly enhanced GFP-Cre degradation.

OVA was then used to construct the pre-fused PROTAC tumor antigen (denoted as pre-fused AHPC-OVA) and characterized by SDS polyacrylamide gel electrophoresis (**FIG. 8D**). Förster resonance energy transfer (FRET) analysis demonstrated the formation of pre-fused AHPC-OVA/pVHL binary complexes. OVA and pVHL were pre-labelled with fluorescein isothiocyanate (FITC) and RITC, respectively. FRET analysis showed effective energy transfer after co-incubating pre-fused AHPC-OVA and pVHL for 2 hours, suggesting that pre-fused AHPC-OVA and pVHL molecules were close to one another. Negligible energy transfer was observed when mixing OVA, AHPC, and pVHL (**FIG. 8E**). These results confirmed the ability of pre-fused AHPC-OVA to recruit pVHL. Then, proteasome-mediated OVA degradation in murine bone marrow-derived dendritic cells (BMDCs) was tested by Western blot. Semi-quantitative analysis showed that intracellular delivery of pre-fused AHPC-OVA by LNPs (denoted as TAgD-TVac<sub>OVA</sub>) exhibited the most OVA degradation compared to delivery of OVA (denoted as OVA LNP) and co-delivered OVA and AHPC (denoted as OVA + AHPC LNP) (**FIG. 2I**). To further confirm the proteasomal degradation, cytoplasmic OVA levels were assessed. Significantly reduced OVA level was observed in the TAgD-TVac<sub>OVA</sub> group, confirming enhanced OVA degradation (**FIG. 8F**). The cross-presentation of OVA fragments in BMDCs was assessed by flow cytometry and CLSM. As shown in **FIGs. 2J & 2K**, BMDCs treated with TAgD-TVac<sub>OVA</sub> for 12 hours exhibited the most efficient cross-presentation of OVA fragments on MHC-I, as demonstrated by staining H2kb-SIINFEKL complex. Extending the incubation time to 24 hours further increased cross-presentation. These results were confirmed by immunofluorescence analysis. The highest H2kb-SIINFEKL complex signal (green) was observed in the TAgD-TVac<sub>OVA</sub> group (**FIG. 9A**). We also found that TAgD-TVac<sub>OVA</sub>-treated BMDCs showed significantly upregulated expression of co-stimulatory markers CD80 and CD86, suggesting enhanced DC maturation (**FIG. 9B & 9C**). These results indicated that TAgD-TVac<sub>OVA</sub> could improve the cross-presentation of tumor antigens in DCs via UPP, which is crucial for cross-priming of cytotoxic T-cells.

### **TAgD-TVac triggers robust cytotoxic T-cell activation through improving tumor antigen cross-presentation**

LNs are important sites for antigen presentation and cytotoxic T-cell stimulation. To achieve robust T-cell cross-priming, pre-fused AHPC-OVA should be delivered into LNs. To demonstrate, pre-fused AHPC-OVA was pre-labelled with Cyanine5.5 NHS ester (Cy5.5) when preparing TAgD-TVac<sub>OVA</sub>. The biodistribution of Cy5.5-labelled TAgD-TVac<sub>OVA</sub> in BALB/c mice was visualized using IVIS imaging system. As demonstrated in **FIGs. 3A & 3B**, compared with LNPs formulated with ALC-0315 (denoted as ALC-0315<sub>OVA</sub>), a key component in the Pfizer-BioNTech COVID-19 Vaccine Comirnaty®, TAgD-TVac<sub>OVA</sub> showed significantly reduced accumulation in the liver and higher accumulation in LNs. After treatment with ALC-0315<sub>OVA</sub>, the radiance of fluorescence in the liver was about 2.2-fold higher than in LNs. However, the intensity in the liver decreased to 36.1% of that in LNs in the TAgD-TVac<sub>OVA</sub> group, confirming excellent LN-targeting ability (**FIG. 3C**). TAgD-TVac<sub>OVA</sub> might enter LNs via lymphatic vessels after SC administration, because fluorescent signals in the lymphatic vessels connected to the LNs (**FIG. 3C**) were observed. Similar result was observed in C57BL/6 mice (**FIG. S7**). Next, the degradation efficiency of OVA within LNs was assessed by Western blot. Significantly reduced OVA levels were observed in the TAgD-TVac<sub>OVA</sub> group compared to OVA LNP and OVA + AHPC LNP groups, suggesting enhanced OVA degradation (**FIGs. 3E & 3F**).

For efficient antigen cross-presentation, OVA needs to undergo proteasomal degradation in APCs. Thus, we explored which cell types in LNs were targeted by TAgD-TVac. Gene-engineered Ai14 mice were employed due to the activatable and stable expression of red fluorescence protein, tdTomato, after cytosolic delivery of Cre recombinase (**FIG. 3G**). Cells expressing fluorescence could be detected by flow cytometry. GFP-Cre was used as an activator and loaded into adjuvant LNPs. As shown in **FIGs. 3H & 11**, administration of GFP-Cre loaded adjuvant LNPs resulted in positive expression of tdTomato in 23.6% DCs and 25.4% macrophages. These results showed that adjuvant LNPs effectively delivered GFP-Cre into the cytoplasm of APCs. The potential mechanism was that adjuvant LNPs were taken up by APCs after injection, then reached LNs through afferent lymphatic vessels via lymphocyte homing. To confirm this hypothesis, Cy5.5-labelled TAgD-TVac<sub>OVA</sub> was injected into a macrophage-depleted mouse model. As demonstrated in **FIG. 3I**, compared to untreated mice, the fluorescent signals in LNs significantly reduced in macrophage-depleted mice. Quantitative results showed that the fluorescence intensities were reduced to 34.5% of the original (**FIG. 3J**). Although the molecular-level mechanism of the biodistribution of TAgD-TVac in mice

remains unclear, our results confirmed the pivotal role of APCs in the LN-targeting ability of TAgD-TVac.

The effects of TAgD-TVac<sub>OVA</sub> on amplifying cross-presentation and inducing anti-tumor T-cell responses were tested in an OVA-expressing melanoma (B16F10-OVA) mouse model. The growth of tumors was monitored after SC vaccination of the mice with phosphate buffered saline (PBS), OVA LNPs, OVA + AHPC LNPs, or TAgD-TVac<sub>OVA</sub> (protein, 20 µg per dose, **FIG. 3K**). As shown in **FIGs. 3L, 3M, and 12A**, treatment with TAgD-TVac<sub>OVA</sub> effectively inhibited the growth of B16F10-OVA tumors. Consistently, significant improvements in survival was observed in TAgD-TVac<sub>OVA</sub>-treated mice (survival rate, 16.7%) during 40 days of observation compared to other treatment groups (0%, **FIG. 3N**). Tumors were then removed for hematoxylin and eosin (H&E) staining (**FIG. 12B**). A drastic reduction in the number of nuclei and significant karyopyknosis were observed in tumors collected from the TAgD-TVac<sub>OVA</sub> group. In contrast, little or moderate apoptosis was observed in other treatment groups. The enhanced anti-tumor efficiency of TAgD-TVac<sub>OVA</sub> should be attributed to the amplified cross-presentation and robust cytotoxic T-cell activation.

For verification, the anti-tumor immunity triggered by TAgD-TVac<sub>OVA</sub> was assessed by flow cytometry. TAgD-TVac<sub>OVA</sub> treatment significantly improved the cross-presentation of OVA fragments (H2Kb-SIINFEKL<sup>high</sup>, 19.2%) onto MHC-I on the surface of DCs in LNs. In contrast, moderate cross-presentation efficiencies were observed in other treatment groups (OVA LNP, 10.1%; OVA + AHPC LNP, 9.76%) (**FIGs. 3O, 13A, and 14A**). Moreover, DCs from TAgD-TVac<sub>OVA</sub>-treated mice exhibited significantly upregulated expression of CD80 and CD86, confirming enhanced DC maturation (**FIGs. 14A & 14F**). The improved OVA cross-presentation and DC maturation should be attributed to superior LN-targeting ability of TAgD-TVac<sub>OVA</sub> and more efficient degradation of pre-fused AHPC-OVA in DCs. Activation of innate immunity was confirmed by assessing the levels of cytokines and chemokines in the blood. As shown in **FIG. 15**, TAgD-TVac<sub>OVA</sub> treatment increased pro-inflammatory cytokine (including M-CSF, IFN-γ, and IL-6) and chemokine (including MCP-1, MIP-1α, and MIP-1β) levels. Among them, IL-6 plays an important role in the proliferation and differentiation of cytotoxic T-cells for adaptive immunity. Thus, the activation of intra-tumoral cytotoxic CD8<sup>+</sup> T-cells was analyzed, which are major effectors for tumor suppression. The highest CD8<sup>+</sup> T-cell infiltration was observed in the TAgD-TVac<sub>OVA</sub> group (CD8<sup>+</sup>, 31.1%), which should be attributed to enhanced antigen cross-presentation in LNs (**FIG. 3P & S14C**). CD8<sup>+</sup> T-cells in the TAgD-TVac<sub>OVA</sub> group showed significantly increased expression of granzyme B (mediating apoptosis, GzmB<sup>high</sup>, 27.8%), further confirming the activation of T-cells (**FIGs.**

**3Q & 11D**). Efficient antigen cross-presentation also induced a strong immunological memory, which was confirmed by assessing the proportions of effector memory T ( $T_{EM}$ ) cells and central memory T ( $T_{CM}$ ) cells in the spleen. Treatment with TAgD-TVac<sub>OVA</sub> led to significant increases in the proportions of CD62L<sup>low</sup>CD44<sup>high</sup>  $T_{EM}$  cells (32.6%) and CD62L<sup>high</sup>CD44<sup>high</sup>  $T_{CM}$  cells (24.8%) in the spleen compared to other treatment groups (**FIGs. 3R, 14E, and 14G**). The crucial roles of T-cells were further confirmed using CD8<sup>+</sup> T-cell-depleted B16F10-OVA tumor-bearing mice. As shown in **FIG. 14H**, significantly reduced anti-tumor effect of TAgD-TVac<sub>OVA</sub> was found.

#### **Anti-tumor effects of TAgD-TVac in a subcutaneous breast cancer model**

Breast cancer is the second most common cancer in women. Thus, we investigated the anti-tumor activity of TAgD-TVacs on 4T1 triple-negative breast cancer model. To mimic the antigens sourced from the patient tumor tissues, tumor lysate proteins (TLP<sub>4T1</sub>) isolated from luciferase-transfected 4T1 (4T1-Luc) tumor cells were employed to prepare the TAgD-TVac (denoted as TAgD-TVac<sub>4T1</sub>). Adjuvant LNPs co-loaded with TLP<sub>4T1</sub> and AHPC acted as the control (denoted as TLP<sub>4T1</sub> + AHPC LNP). Tumor-bearing mice received prime and boost doses of TAgD-TVac<sub>4T1</sub> (protein, 20 µg per dose) on days 0, 5, and 10 (**FIG. 4A**). TAgD-TVac<sub>4T1</sub> treatment effectively suppressed the growth of 4T1-Luc tumors, which was demonstrated by measuring *in vivo* bioluminescence and volumes of 4T1-Luc tumors (**FIGs. 4B, 4C and 4D**). These results were confirmed with histological analysis of H&E-stained tumor sections (**FIG. 15**). As a result, significant improvements in survival were observed in mice treated with TAgD-TVac<sub>4T1</sub> (survival rate, 33.3%) over 40 days of observation, compared to other treatment groups (PBS, 0%; TLP<sub>4T1</sub> + AHPC LNP, 16.7%, **FIG. 4E**). Importantly, it was found that one TAgD-TVac<sub>4T1</sub> treated mouse with no detectable tumor at the end of this experiment (**FIG. 4C**). It might be attributed to abundant tumor antigens in tumor cell lysate, which elicited more efficient anti-tumor immunity.

Next, the anti-tumor immunity elicited by TAgD-TVac<sub>4T1</sub> was investigated. First, DC maturation in LNs was studied by measuring CD80 and CD86, co-stimulatory markers important for cross-priming. As shown in **FIGs. 4F & 11A**, TAgD-TVac<sub>4T1</sub> treatment significantly upregulated CD80 and CD86 expression (CD80<sup>high</sup>CD86<sup>high</sup>, 20.4%) on DCs. In contrast, moderate CD80 and CD86 expression was observed in the TLP<sub>4T1</sub> + AHPC LNP group (9.75%). These results confirmed TAgD-TVac<sub>4T1</sub>-induced DC maturation, which is crucial for efficient cytotoxic T-cell cross-priming. The activation of intra-tumoral cytotoxic CD8<sup>+</sup> T-cells was then analyzed. Compared to other treatment groups, the highest CD8<sup>+</sup> T-cell

infiltration was observed in the TAgD-TVac<sub>4T1</sub> group (CD8<sup>+</sup>, 32.1%) (**FIGs. 4G** and **11B**). TAgD-TVac<sub>OVA</sub> treatment also significantly increased granzyme B expression (GzmB<sup>high</sup>, 19.1%) by CD8<sup>+</sup> T-cells (**FIG. 4H** and **FIG. 17C**). These results confirmed cytotoxic T-cell-mediated anti-tumor immunity after SC vaccination using TAgD-TVac<sub>4T1</sub>.

Vaccination by TAgD-TVac<sub>4T1</sub> also triggered strong immunological memory. Treatment with TAgD-TVac<sub>4T1</sub> resulted in elevated proportions of CD62L<sup>low</sup>CD44<sup>high</sup> T<sub>EM</sub> cells (31.6%) and CD62L<sup>high</sup>CD44<sup>high</sup> T<sub>CM</sub> cells (34.4%) in the spleen, compared to TLP<sub>4T1</sub> + AHPC LNPs (T<sub>EM</sub> cells, 23.0%; T<sub>CM</sub> cells, 26.9%) (**FIGs. 4I, 17D, and 17E**). Considering the crucial role of memory T-cells in preventing tumor metastasis, we evaluated the effects of TAgD-TVac<sub>4T1</sub> on inhibiting tumor metastasis using IVIS imaging system. 4T1-Luc tumor-bearing mice in TLP<sub>4T1</sub> + AHPC LNP and TAgD-TVac<sub>4T1</sub> groups were rechallenged with 4T1-Luc cells via IV injection. A control group (normal mice) was also treated with 4T1-Luc cells via IV injection. As demonstrated in **FIGs. 4K & 4L**, treatment with TAgD-TVac<sub>4T1</sub> effectively inhibited tumor metastasis. In contrast, mice who received other treatments all exhibited significant lung metastasis. Histological analysis of H&E-stained lung sections confirmed this result. TAgD-TVac<sub>4T1</sub> treatment resulted in significant reduction of 4T1-Luc tumor nodules in lung tissues compared to other treatment groups (**FIG. 16**). These results indicated that TAgD-TVac<sub>4T1</sub> could induce efficient T-cell-dependent anti-tumor immunity for the inhibition of primary and metastatic tumor growth. Anti-tumor effects of TAgD-TVac in an orthotopic lung cancer model

Lung cancer (carcinoma) is the third most common cancer in the United States. The potential of TAgD-TVac to suppress orthotopic lung cancer development in mice was investigated, and it demonstrated its ability to improve therapeutic outcomes of ICB therapy. Luciferase stably expressed Lewis lung carcinoma (LLC-Luc) cells were intercostally inoculated in the right lung of C57BL/6 mice. Tumor lysate proteins isolated from LLC-Luc cells were employed to prepare the TAgD-TVac (denoted as TAgD-TVac<sub>LLC</sub>). Tumor-bearing mice received three doses of TAgD-TVac<sub>LLC</sub> (protein, 20 µg per dose) on days 0, 4, and 8. Anti-PD-1 antibodies (aPD-1, 200 µg per dose) were administered by intraperitoneal (IP) injection on days 1, 5, and 9 (**FIG. 5A**). The anti-tumor effect of single administration of TAgD-TVac<sub>LLC</sub> was also evaluated. Single administration of a PD-1 inhibitor was used for comparison. Since LLC-Luc tumors are expected to develop in the lungs, we do not expect any externally visible tumor masses, so we rely on body weight to assess tumor progression. As shown in **FIG. 5B**, treatment with TAgD-TVac<sub>LLC</sub> significantly inhibited the growth of LLC-Luc tumors. TAgD-TVac<sub>LLC</sub> treatment coupled with aPD-1 (denoted as aPD-1 + TAgD-

TVac<sub>LLC</sub>) further suppressed tumor development. Then, lung tissues were collected. As demonstrated in **FIGs. 5C & 5D**, significant reduction of LLC-Luc tumor nodules in the lungs were observed after receiving aPD-1 + TAgD-TVac<sub>LLC</sub> treatment. This result was confirmed with histological analysis of H&E stained lung sections (**FIG. 18**). As a result, TAgD-TVac<sub>LLC</sub> treatment coupled with aPD-1 effectively reduced the decline of body weight and prolonged the survival of tumor-bearing mice over 40 days of observation (survival rate, 50.0%, **FIG. 5E** and **5F**). The enhanced ICB therapy should be attributed to TAgD-TVac<sub>LLC</sub>-triggered DC activation in LNs.

For verification, the activation of DCs in LNs was explored by measuring CD80 and CD86. Compared to single administration of aPD-1 (CD80CD86<sup>high</sup>, 6.17%) or TAgD-TVac<sub>LLC</sub> (7.24%), treatment with TAgD-TVac<sub>LLC</sub> significantly upregulated expression of CD80 and CD86 (12.9%), confirming enhanced DC maturation (**FIGs. 5G & S19A**). The level of IL-6 in the blood was assessed due to its crucial role in T-cell-mediated adaptive anti-tumor immunity. Both aPD-1 and TAgD-TVac<sub>LLC</sub> could increase the levels of IL-6. Combination therapy of aPD-1 and TAgD-TVac<sub>LLC</sub> showed a 2.2-fold increase in IL-6 levels compared to aPD-1 alone, confirming a robust T-cell activation (**FIG. 5H**). The establishment of immunological memory was then evaluated. As shown in **FIGs. 5I, 19A, and 19C**, treatment with TAgD-TVac<sub>LLC</sub> improved proportions of CD62L<sup>low</sup>CD44<sup>high</sup> T<sub>EM</sub> cells (23.6%) and CD62L<sup>high</sup>CD44<sup>high</sup> T<sub>CM</sub> cells (27.1%) in the spleen. Further combined with aPD-1, the proportions of CD62L<sup>low</sup>CD44<sup>high</sup> T<sub>EM</sub> cells and CD62L<sup>high</sup>CD44<sup>high</sup> T<sub>CM</sub> cells in the spleen were increased to 37.1% and 33.2%, respectively, indicating stronger immunological memory.

T-cell-dependent adaptive immunity shaped by APCs is antigen specific. To demonstrate, LLC-Luc tumor-bearing mice that have received aPD-1 + TAgD-TVac<sub>LLC</sub> combination therapy were rechallenged with LLC-Luc (left flank) and non-cognate B16F10 tumor cells (right flank) via SC injection on day 40 to mimic tumor recurrence (**FIGs. 5J & 5K**). A control group (normal mice) was also treated with LLC-Luc and B16F10 tumor cells by SC injection. As demonstrated in **FIG. 5L & 5M**, LLC-Luc tumor recurrence was completely suppressed in two out of five mice in the aPD-1 + TAgD-TVac<sub>LLC</sub> group. In sharp contrast, slight B16F10 tumor growth suppression was observed. Selective suppression of LLC-Luc tumor growth was attributed to activation of T-cells, as evidenced by detection of intra-tumoral CD8<sup>+</sup> T-cell infiltration (CD8<sup>+</sup>, 20.9%; Control, 5.37%, **FIGs. 19D & 19E**). These results indicated that TAgD-TVac<sub>LLC</sub> combined with ICB therapy effectively induced protective cognate anti-tumor immune response, confirming robust T-cell-dependent adaptive

immunity and strong immunological memory. Anti-tumor effects of TAgD-TVac in an inoperable ovarian cancer model.

Ovarian cancer is a high-grade serious tumor. Due to the lack of typical clinical symptoms in the early stage, many patients are accompanied by intra-abdominal organ metastases when they are diagnosed, which makes surgical treatment impossible. In view of this clinical challenge, an advanced ovarian cancer model was established to test the therapeutic outcomes of our developed TAgD-TVac technology. In this study, the TAgD-TVac was formulated using tumor lysate proteins isolated from luciferase stably expressed ovarian (ID8-Luc) tumor cells (denoted as TAgD-TVac<sub>ID8</sub>). The tumor-bearing mice received three doses of TAgD-TVac<sub>ID8</sub> (protein, 20 µg per dose) on days 0, 4, and 8, and additional three doses of aPD-1 (200 µg per dose) on days 1, 5, and 9 (**FIG. 6A**). As shown in **FIGS. 6B & 6F**, treatment with TAgD-TVac<sub>ID8</sub> effectively suppressed the growth of intra-abdominal tumors, and its effect was slightly better than ICB monotherapy. Combination therapy of TAgD-TVac<sub>ID8</sub> with aPD-1 (denoted as aPD-1 + TAgD-TVac<sub>ID8</sub>) exhibited more significant tumor growth inhibition than aPD-1 or TAgD-TVac<sub>ID8</sub> administration alone. Then, bioluminescence signals in the abdominal cavity and in each major organ were analyzed. As demonstrated in **FIG. 6C**, significant reduction of ID8-Luc tumor nodules in the abdominal cavity (and peritoneum), ovarian, and spleen were observed after the mice receiving aPD-1 + TAgD-TVac<sub>ID8</sub> treatment. This result was confirmed by histological analysis and photos (**FIGS. 6D, 6E, 20A, and 20B**). Consistently, TAgD-TVac<sub>ID8</sub> prolonged the survival rate of the tumor-bearing mice during 80 days of observation, which was longer than the PBS group (**FIG. 6G**). Combination with aPD-1 further improved survival rate, and 66.7% of mice were still alive. Ascites volume was also significantly decreased in aPD-1 + TAgD-TVac<sub>ID8</sub>-treated mice (**FIG. 6H**). These results demonstrated that combination of ICB therapy with TAgD-TVac strategy could synergistically enhance the anti-tumor efficiency. The anti-tumor immunity triggered by TAgD-TVac<sub>ID8</sub> and combination therapy were then assessed. As shown in **FIGS. 6I & 20C**, TAgD-TVac<sub>ID8</sub> (CD80<sup>high</sup>CD86<sup>high</sup>, 11.3%) and combination therapy (15.0%) effectively enhanced the activation of DCs in LNs. We also found that TAgD-TVac<sub>ID8</sub> treatment increased the levels of pro-inflammatory cytokines (including M-CSF, IFN-γ, and IL-6, **FIGS. 6J-6L**) and pro-inflammatory chemokines (including MCP-1, MIP-1α, and MIP-1β, **FIG. 6M-6O**) in the blood, confirming the activation of innate immunity. Innate immunity activation was further improved through combination with aPD-1. These cytokines and chemokines would trigger the proliferation and differentiation of T-cells for robust adaptive anti-tumor immunity.

## Summary

A TAgD-TVac was developed by loading pre-fused PROTAC tumor antigens into an adjuvant LNP with LN-targeting ability. TAgD-TVac delivered the pre-fused PROTAC tumor antigens into APCs and then to the UPP under the guidance of E3 ligase ligands, which amplified MHC-I-dependent antigen cross-presentation and initiated cytotoxic T-cell activation within LNs. In Four personalized TAgD-TVacs were prepared using relevant tumor lysates, and successfully demonstrated their capability to elicit potent antigen-specific adaptive immunity and immunological memory against multiple solid tumors. Combining TAgD-TVac with ICB therapy further inhibited tumor growth and reduced the risk of tumor metastasis and recurrence, which holds translational potential. Current efforts provide a novel route to initiate an adaptive immune response via simple chemistry and nanotechnology, informing the design of effective cancer immunotherapies. The success in PROTAC-based vaccine technology may also represent an important breakthrough at the interface between basic research of ubiquitin–proteasome proteolytic systems and vaccine development.

## Materials and Methods

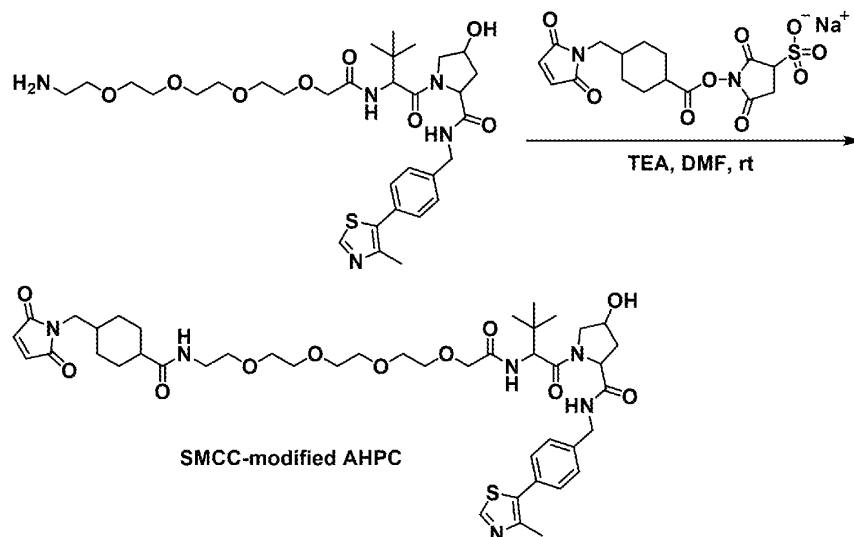
Unless otherwise noted, reagents and solvents were used as received from commercial sources without further purification. All the reagents and solvents for 113O-12B lipid synthesis were purchased from Sigma-Aldrich or Fisher Scientific. 2-aminothiophene (Traut's reagent), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), and tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl) were purchased from Fisher Scientific. Protamine sulfate salt (from salmon) was purchased from Sigma-Aldrich. Ovalbumin was purchased from Abnova. (S,R,S)-AHPC-PEG<sub>4</sub>-amine was purchased from Broadpharm. Von Hippel Lindau Recombinant Protein was purchased from Novus Biologicals™. Fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RhB) were purchased from Sigma-Aldrich. 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) was purchased from Invitrogen. Cholesterol (Chol), D-Luciferin potassium salt, and Cell Counting Kit-8 (CCK-8) were purchased from Thermo Scientific™. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG) were purchased from Avanti Polar Lipids. Carbon support film 5-6 nm thick on Square 200 mesh Copper Grid was purchased from Electron Microscopy Sciences. Bicinchoninic acid disodium salt hydrate (BCA) was purchased from Solarbio. Dulbecco's Modified Eagle Medium (DMEM) growth medium, Fetal Bovine Serum (FBS), and Dulbecco's phosphate buffered saline solution (D-PBS, 10 mM, pH 7.2)

were purchased from Gibco (Gibco Corporation). Trypsin-EDTA (0.25%), and LysoTracker Red were purchased from Invitrogen. B16F10, 4T1-Luc, LLC-Luc, ID8-Luc tumor cell lines, and DC2.4 cell line were purchased from American type culture collection (ATCC). Lysosome Enrichment Kit for Tissues and Cultured Cells was purchased from Thermo Scientific™. The antibodies for western blot analysis, flow cytometry, and confocal laser scanning microscope observation (CLSM) were purchased from Invitrogen (Thermo Fisher Scientific), Cell Signaling Technology (USA), and Biolegend (USA).

#### Instrument

All  $^1\text{H}$  nuclear magnetic resonance ( $^1\text{H}$  NMR) spectra were recorded on a Bruker AVIII 500 MHz NMR spectrometer operated in the Fourier transform mode. ESI-MS spectra was collected by Finnigan LTQ. Hydrodynamic size and polydispersity index of nanoparticles were measured by a Zeta-PALS particle size analyzer (Brookhaven Instruments). Transmission Electron Microscopy (TEM) measurements were performed on a FEI Technai Spirit Transmission Electron Microscope. The cells were observed using confocal laser scanning microscopy (Eclipse Ti2, Nikon, Japan). Flow cytometry analysis was performed on a BD Accuri C6 flow cytometry or a LSR-II flow cytometer (BD, USA). *In vivo* imaging of the mice was performed on PerkinElmer® IVIS® Systems (Bioluminescence Imaging, Boston, USA).

#### Preparation of pre-fused AHPC-GFP-Cre and pre-fused AHPC-OVA



**Scheme 1** | Synthesis of SMCC-modified AHPC.

*Preparation of pre-fused AHPC-GFP-Cre*

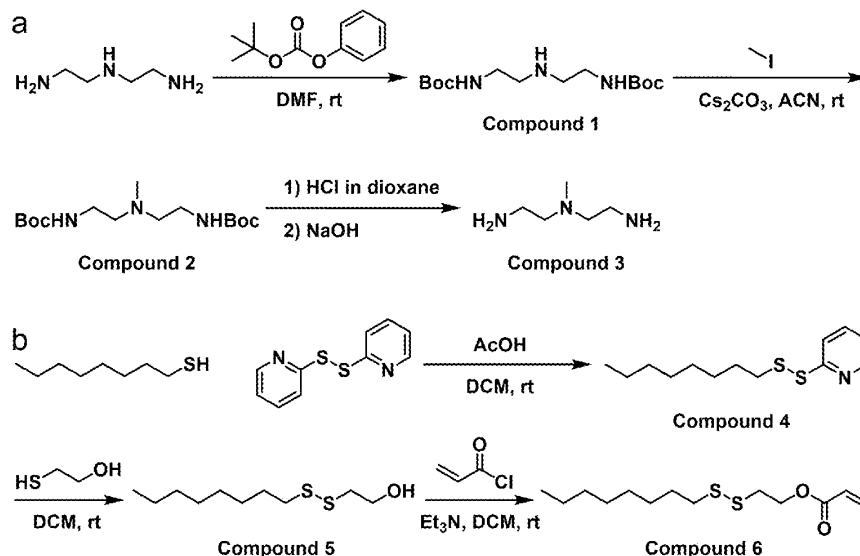
GFP-Cre (0.5 mg) was dissolved in 0.5 mL phosphate buffered saline solution (PBS, 10 mM, pH 7.4), then Traut's Reagent (5 equiv) and tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl, 10 equiv) was added. The solution was stirred at room temperature for 3 hours, and then dialyzed against PBS at 4 °C to obtain thiol-modified GFP-Cre.

(*S,R,S*)-AHPC-PEG<sub>4</sub>-amine (20 mg, 0.03 mmol) was dissolved in 0.4 mL dimethylformamide (DMF) with triethylamine (TEA, 0.06 mmol, 2 equiv). Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) (28 mg, 0.06 mmol) dissolved in 0.2 mL DMF was added dropwise to the stirred mixture and reacted at room temperature for 24 hours. The solvent was then evaporated under reduced pressure and the residue was purified by silica gel column chromatography (dichloromethane/methanol, 10/1, v/v) to yield 22 mg of SMCC-modified AHPC (0.02 mmol, 77.9% yield) as a white solid. TLC: *R<sub>f</sub>* = 0.51 (dichloromethane/methanol, 6/1, v/v). ESI-MS: *m/z* [M + Na<sup>+</sup>] C<sub>57</sub>H<sub>111</sub>N<sub>3</sub>O<sub>8</sub>S<sub>8</sub> calculated: 905.42; found: 905.64.

Thiol-modified GFP-Cre and SMCC-modified AHPC (15/1, w/w) were mixed in PBS, then TCEP (thiol-modified GFP-Cre/TCEP, 1/10, n/n) was added and stirred at room temperature for 8 hours. The mixture was dialyzed in PBS to obtain purified pre-fused AHPC-GFP-Cre and stored at 4 °C for further use.

*Preparation of pre-fused AHPC-OVA*

The pre-fused AHPC-OVA was prepared in a similar method and assessed by SDS polyacrylamide gel electrophoresis.

*Synthesis of 113-O12B lipid***Scheme 2** | Synthesis of Compound 3 and Compound 6.*Synthesis of Compound 3*

Compound 1 was synthesized according to previous literature<sup>1</sup>. A solution of Compound 1 (0.6 g, 2 mmol) in 10 ml of acetonitrile (ACN), cesium carbonate (2.0 g, 6 mmol) and iodomethane (0.15 mL, 2.4 mmol) was added under an argon atmosphere and stirred overnight at room temperature. The solvent was removed under reduced pressure. The crude Compound 2 was then dissolved in a mixture of ethyl acetate (10 mL) and water (10 mL). The aqueous layer was extracted twice with ethyl acetate (10 mL). The combined organic layers were dried with anhydrous magnesium sulfate ( $\text{MgSO}_4$ ), filtered off and the solvent was removed in reduced pressure. The residue was purified by silica gel column chromatography (dichloromethane:methanol, 95/5 to 90/10, v/v) to yield 0.5 g of **Compound 2** (1.6 mmol, 78.1% yield). TLC:  $R_f = 0.68$  (dichloromethane/methanol, 6/1, v/v). ESI-MS:  $m/z$  [ $\text{M} + \text{H}^+$ ]  $\text{C}_{15}\text{H}_{31}\text{N}_3\text{O}_4$  calculated: 318.23; found: 318.55.

A solution of Compound 2 (1.0 g, 3 mmol) in 10 mL of ethyl acetate, was added dropwise 4N HCl in dioxane (4.5 ml, 18 mmol) at 4 °C under an argon atmosphere. The reaction mixture was stirred at room temperature for 12 hours and the solvent was removed under reduced pressure. The residue was triturated with diethyl ether three times and a small amount of ethyl acetate. The resultant solids were dissolved in 10N NaOH and extracted with dichloromethane (6 × 20 mL). The combined organic layers were dried with anhydrous  $\text{MgSO}_4$ ,

filtered off and the solvent was removed to yield 0.3 mg of **Compound 3** (0.026 mmol, 85.4% yield). ESI-MS:  $m/z$   $[M + H^+]$   $C_5H_{15}N_3$  calculated: 118.13; found: 118.28.

#### *Synthesis of 113-O12B lipid*

Compound 3 (head amine) and Compound 6 (bio reducible acrylate-modified alkyl chains) were mixed and heated (70 °C) at 1:4.8 molar ratio in Teflon-lined glass screw-top vials for 48 hours. The crude product was purified by silica gel column chromatography to yield **113-O12B lipid**.  $^1H$  NMR ( $CDCl_3$ , 500 MHz, ppm): 4.35 (t,  $J=5.0$  Hz, 8H), 2.92 (t,  $J=5.0$  Hz, 8H), 2.82 (t,  $J=5.0$  Hz, 8H),  $\delta$  2.72 (t,  $J=5.0$  Hz, 8H), 2.58 (t,  $J=5.0$  Hz, 4H), 2.51–2.45 (m, 12H), 2.26 (s, 3H), 1.69 (t,  $J=5.0$  Hz, 8H), 1.40 (t,  $J=5.0$  Hz, 8H), 1.34–1.27 (m, 32H), 0.93–0.89 (m, 12H).  $^{13}C$  NMR ( $CDCl_3$ , ppm):  $\delta$  172.3, 62.6, 56.2, 51.9, 49.6, 43.1, 39.3, 37.1, 32.6, 31.8, 29.7, 29.2, 29.1, 28.5, 22.6, 14.1. ESI-MS:  $m/z$  calculated for  $[M + H^+]$   $C_{57}H_{111}N_3O_8S_8$ : 1221.61; found: 1222.23.

#### *Preparation of adjuvant LNP*

##### *Preparation of adjuvant LNP*

Adjuvant LNPs were formulated with adjuvant lipidoids (113-O12B), cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG<sub>2k</sub>) at a molar ratio of 48.5:38:10:3.5 using the ethanol dilution method.

For the preparation of DiR-labelled adjuvant LNP, we dissolved the mixture of total lipids and DiR at a weight ratio of 20/1 in ethanol. 100  $\mu$ L of above solution was then added dropwise to 300  $\mu$ L of sodium acetate buffer (25 mM, pH 5.2) and vortexed vigorously. Next, we removed the ethanol in the formulation by dialysis against PBS (molecular weight cutoff, 35 kDa) for 12 hours. The obtained DiR-labelled adjuvant LNP was stored at 4 °C for further use (protected against exposure to light).

##### *Preparation of pre-fused AHPC-GFP-Cre LNP, GFP-Cre-loaded adjuvant LNP, and TAgD-TVac*

For the preparation of different protein-loaded LNP formulations, we mixed different protein payloads with adjuvant LNPs at a weight ratio of 1/10 and vortexed vigorously. Then solution was then incubated at room temperature for 5 min. The obtained formulations were stored at 4 °C for further use.

***Characterization of adjuvant LNP and TAgD-TVac******TEM measurements***

To prepare the TEM sample, 10  $\mu$ L sample solution was dropped onto a carbon-coated copper grid for 10~15 min and blotted with filter paper to remove excess liquid. Then the sample was negatively stained with phosphotungstic acid (EPTA) (5~10  $\mu$ L) for 1~2 min, blotted again and air-dried before analysis on TEM.

***DLS measurements***

The hydrodynamic size of different formulations was evaluated in PBS at 37 °C.

***LN-targeting capability of adjuvant LNP***

All animal procedures were performed with ethical compliance and approval by the Institutional Animal Care and Use Committee at the Tufts University (Animal Protocol# M2023-33). LN-targeting ability of adjuvant LNPs was evaluated in BALB/c and C57BL/6 mice using DiR-labelled formulation. 50  $\mu$ g of DiR-labelled adjuvant LNPs were subcutaneously injected into the mice at the tail base. After 6 hours, the biodistribution of DiR-labelled adjuvant LNPs was visualized using the IVIS imaging system (PerkinElmer, Boston, MA).

***The ability of pre-fused AHPC-GFP-Cre to target intracellular pVHLs***

Adjuvant LNPs were employed to deliver pre-fused AHPC-GFP-Cre into DC 2.4 cells (denoted this formulation as pre-fused AHPC-GFP-Cre LNP). Flow cytometry and confocal laser scanning microscope (CLSM) observation were used to study cellular uptake of pre-fused AHPC-GFP-Cre LNPs and the ability of pre-fused AHPC-GFP-Cre to target intracellular pVHLs.

For flow cytometry assays, DC 2.4 cells ( $5 \times 10^4$  cells per well) were seeded in a 12-well plate and incubated overnight for cell attachment. Different formulations including pre-fused AHPC-GFP-Cre LNPs and GFP-Cre + AHPC LNPs (GFP-Cre, 2  $\mu$ g/mL; adjuvant LNP, 20  $\mu$ g/mL) were incubated with cells for 3 hours, then the cells were washed with PBS three times, followed by digesting using 0.25% trypsin for flow cytometry analysis.

For CLSM observation, DC 2.4 cells were seeded in confocal dishes and allowed to grow until approximately 80% confluence. Different formulations including pre-fused AHPC-GFP-Cre LNPs and GFP-Cre + AHPC LNPs (GFP-Cre, 2  $\mu$ g/mL; adjuvant LNP, 20  $\mu$ g/mL) were incubated with cells for 3 hours, and then replaced by fresh culture medium for additional 9-hour incubation. The cells were washed with PBS three times and fixed using 4%

paraformaldehyde. Intracellular pVHLs were stained by anti-VHL antibodies and fluorescent secondary antibodies (red). The nucleus was stained with DAPI (blue).

***TAgD-TVac<sub>OVA</sub>-mediated proteasomal degradation of OVA and antigen cross-presentation***

Bone marrow-derived dendritic cells (BMDCs) were generated from the bone marrow of C57BL/6 mice.

Western blotting was performed to assess cellular OVA levels in BMDCs. For western blots, BMDCs ( $1 \times 10^5$  cells per well) were seeded in a 6-well plate and incubated overnight for cell attachment. Different formulations (OVA, 20  $\mu\text{g/mL}$ ; adjuvant LNP, 200  $\mu\text{g/mL}$ ) were incubated with cells for 3 hours, and then replaced by fresh culture medium for additional 24-hour incubation. The cells were then washed with PBS three times and lysed in 0.5 mL of lysis buffer (PBS containing 1% Triton X-100 and 1 mM PMSF) at 4 °C for 30 min. Equal amounts protein loadings were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane. Nonspecific binding sites were blocked with 5% nonfat milk in PBS and 0.05% Tween 20 (PBST) for 2 hours at room temperature. Membranes were incubated in the primary antibodies at appropriate concentrations for 24 hours at 4 °C overnight, followed by three times rinse with PBST buffer for 15 min. The membranes were then incubated in the secondary antibodies (1:10000) for 1 hour at room temperature. The membranes were detected and analyzed using a computerized chemiluminescent imaging system. To evaluate the levels of OVA in cytoplasm, the cytosolic fractions were prepared using a Lysosome Enrichment Kit. Antibody list: anti-chicken ovalbumin antibody (Invitrogen, Catalog# PA1196, clone# Polyclonal), anti-mouse GAPDH antibody (Novus Biologicals, Catalog# NB300326, clone# Polyclonal).

The BMDCs were incubated with different formulations (OVA, 20  $\mu\text{g/mL}$ ; adjuvant LNP, 200  $\mu\text{g/mL}$ ) for 12 hours or 24 hours. BMDCs were then collected and stained with fluorescent conjugated antibodies for flow cytometry analysis. Antibody list: PE-anti-mouse CD11C antibody (BioLegend, Catalog# 117308, clone# N418), APC-anti-mouse H2kb-SIINFEKL antibody (eBioscience, Catalog# 17-5743-82, clone# eBio25-D1.16), FITC-anti-mouse CD80 antibody (BD Biosciences, Catalog# 561954, clone# 16-10A1), and APC-anti-mouse CD86 antibody (BioLegend, Catalog# 105012, clone# GL-1).

***LN-targeting ability of TAgD-TVac<sub>OVA</sub>***

The pre-fused AHPC-OVA was pre-labelled with Cyanine5.5 NHS ester (Cy5.5) for the preparation of TAgD-TVac<sub>OVA</sub>. LN-targeting ability of Cy5.5-labelled TAgD-TVac<sub>OVA</sub> was evaluated in BALB/c and C57BL/6 mice. Cy5.5-labelled TAgD-TVac<sub>OVA</sub> (OVA, 10  $\mu\text{g}$ ;

adjuvant LNP, 100 µg per dose) was subcutaneously injected into the mice at the tail base. After 6 hours, the biodistribution of Cy5.5-labelled TAGD-TVac<sub>OVA</sub> was visualized using the IVIS imaging system.

***Delivery of GFP-Cre to LNs in Ai14 mice using adjuvant LNP***

Ai14 mice were subcutaneously injected with GFP-Cre-loaded adjuvant LNPs (GFP-Cre, 20 µg; adjuvant LNP, 200 µg per dose) at tail base twice (time points, 0 hour and 24 hour). Forty-eight hours after the last injection, the mice were euthanized and inguinal LNs were collected. The cell suspensions were prepared by grinding and filtrating through a 70 µm strainer. Then,  $2 \times 10^6$  cells were incubated in 100 µL flow cytometry staining buffer containing fluorescent conjugated antibodies at the recommended concentrations on the ice for 1 hour. Then, the cells were kept at 4 °C for flow cytometry analysis after washing twice with PBS. Antibody list: APC/Cy7-anti-mouse CD49b antibody (BioLegend, Catalog# 108920, clone# DX5), APC/Cy7-anti-mouse CD3 antibody (BioLegend, Catalog# 100222, clone# 17A2), Brilliant Violet 650-anti-mouse B220 antibody (BioLegend, Catalog# 103241, clone# RA3-6B2), PE/Cy7-anti-mouse CD11C antibody (BioLegend, Catalog# 117318, clone# N418), and PerCP/Cy5.5-anti-mouse F4/80 antibody (BioLegend, Catalog# 123128, clone# BM8).

To establish the macrophage-depleted mouse model, Clodrosome® (10 mL/kg) was injected to the mice to deplete macrophages. Clodrosome® is a well-characterized liposomal drug that has been used to deplete macrophages either in local tissues or peripheral blood, which depends on the injection routes. Briefly, the mice were subcutaneously treated with Clodrosome® 18 hours before administration of Cy5.5-labelled TAGD-TVac<sub>OVA</sub>. The mice were then euthanized and inguinal LNs were collected after 6 hours. Cy5.5 signals in LNs were visualized using the IVIS imaging system.

***The effects of TAGD-TVac<sub>OVA</sub> on inducing anti-tumor T cell responses in vivo***

The female C57BL/6 mice (Charles River) at 6~8-week old and B16F10 tumor cells expressing ovalbumin (B16F10-OVA) were used in this study. Tumor-bearing mice were established by subcutaneous (SC) injection of B16F10-OVA tumor cells ( $1 \times 10^6$  per mouse) into the left flank of C57BL/6 mice. Then, the mice were randomly divided into four groups ( $n = 6$ ) after six days of inoculation. The growth of tumors was monitored after SC vaccination of the mice by phosphate buffered saline solution (PBS), OVA LNP, OVA + AHPC LNP and TAGD-TVac<sub>OVA</sub> at the tail base, respectively (protein, 20 µg per dose; LNP, 200 µg per dose; three doses). The tumor volume (V) was calculated according to the formula:  $V = L \times W^2/2$ , where L and W were the longest and shortest diameter (mm) of the tumor, respectively. The

mice were euthanized when exhibiting signs of impaired health or when the volume of the tumor exceeded 1.5 cm<sup>3</sup>.

For flow cytometry assays, freshly harvested tumor tissues were digested with collagenase IV and made into single-cell suspensions according to the manufacturer's instructions. The tumor cell suspensions were prepared by grinding and filtrating through a 70 µm strainer. Then,  $2 \times 10^6$  cells were incubated in 100 µL flow cytometry staining buffer containing fluorescent conjugated antibodies at the recommended concentrations on the ice for 1 hour for flow cytometry analysis. Antibody list: APC/Cy7-anti-mouse CD45 antibody (BioLegend, Catalog# 103116, clone# 30-F11), APC-anti-mouse CD3 antibody (BD Biosciences, Catalog# 565643, clone# 17A2), PE-anti-mouse CD8 antibody (BD Biosciences, Catalog# 553032, clone# 53-6.7), FITC-anti-mouse CD4 antibody (BD Biosciences, Catalog# 553730, clone# GK1.5), and FITC-anti-mouse GzmB antibody (BioLegend, Catalog# 515403, clone# GB11).

Similar manipulations were performed to analyze immune activation in lymph nodes and spleen using fluorescent conjugated antibodies. Antibody list: PE-anti-mouse-CD11C antibody (BioLegend, Catalog# 117308, clone# N418), APC-anti-mouse-H2kb-SIINFEKL antibody (eBioscience, Catalog# 17-5743-82, clone# eBio25-D1.16), FITC-anti-mouse CD80 antibody (BD Biosciences, Catalog# 561954, clone# 16-10A1), and APC-anti-mouse CD86 antibody (BioLegend, Catalog# 105012, clone# GL-1), APC-anti-mouse CD3 antibody (BD Biosciences, Catalog# 565643, clone# 17A2), PE-anti-mouse CD8 antibody (BD Biosciences, Catalog# 553032, clone# 53-6.7), APC/Cy7-anti-mouse CD62L antibody (BioLegend, Catalog# 104428, clone# MEL-14), and FITC-anti-mouse CD44 antibody (BioLegend, Catalog# 163606, clone# QA19A43).

The levels of pro-inflammatory cytokines (including M-CSF, IFN- $\gamma$ , and IL-6) and pro-inflammatory chemokines (including MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) in the blood were measured with ELISA kits according to the manufacturer's instructions.

For the depletion of CD8<sup>+</sup> T-cells *in vivo*, anti-CD8a antibodies (aCD8, 300 µg per mouse, BioXcell, Catalog# BE0061, clone# 2.43) were intraperitoneally injected.

#### ***Anti-tumor effects of TAgD-TVac<sub>4T1</sub> in a breast cancer mouse model***

Tumor lysate protein solution was prepared via a freeze-thaw process. The cultured 4T1-Luc tumor cells were frozen quickly on dry ice for 5 min and immediately thawed at 37°C. The solution was then vortexed to mix well. This procedure was repeated twice. Protamine sulfate (from salmon) was added to the obtained solution to bind and remove the nucleic acids.

The final solution was centrifugated at 13,000 rpm for 10 min. The supernatant was collected, and the concentration of lysate was measured by bicinchoninic acid (BCA) assay method.

The female BALB/c mice (Charles River) at 6~8-week old and luciferase-transfected 4T1 (4T1-Luc) tumor cells were used in this study. Tumor-bearing mice were established by SC injection of 4T1-Luc tumor cells ( $1 \times 10^6$  per mouse) into the left flank of BALB/c mice. Then, the mice were randomly divided into three groups ( $n = 6$ ) after six days of inoculation. The growth of tumors was monitored after SC vaccination of the mice by PBS, TLP<sub>4T1</sub> + AHPC LNP and TAgD-TVac<sub>4T1</sub> at the tail base, respectively (protein, 20  $\mu$ g per dose; LNP, 200  $\mu$ g per dose; three doses). Tumor growth was monitored by measuring the *in vivo* bioluminescence using the IVIS imaging system (every five days). The tumor volume (V) was calculated according to the formula:  $V = L \times W^2/2$ . The mice were euthanized when exhibiting signs of impaired health or when the volume of the tumor exceeded 1.5 cm<sup>3</sup>.

For flow cytometry assays, freshly harvested tumor tissues were digested with collagenase IV and made into single-cell suspensions according to the manufacturer's instructions. The tumor cell suspensions were prepared by grinding and filtrating through a 70  $\mu$ m strainer. Then,  $2 \times 10^6$  cells were incubated in 100  $\mu$ L flow cytometry staining buffer containing fluorescent conjugated antibodies at the recommended concentrations on the ice for 1 hour for flow cytometry analysis (including APC-Cy7-anti-mouse CD45 antibody, APC-anti-mouse CD3 antibody, PE-anti-mouse CD8 antibody, FITC-anti-mouse CD4 antibody, and FITC-anti-mouse GzmB antibody).

Similar manipulations were performed to analyze immune activation in lymph nodes and spleen using fluorescent conjugated antibodies, including PE-anti-mouse-CD11C antibody, FITC-anti-mouse-CD80 antibody, APC-anti-mouse CD86 antibody, APC-anti-mouse CD3 antibody, PE-anti-mouse CD8 antibody, APC-Cy7-anti-mouse CD62L antibody, and FITC-anti-mouse CD44 antibody.

The mice in TLP<sub>4T1</sub> + AHPC LNP and TAgD-TVac<sub>4T1</sub> groups without obvious tumors were rechallenged with 4T1-Luc tumor cells ( $1 \times 10^6$  cells per mouse) via intravenous (IV) injection on day 18. A PBS control group was also treated with 4T1-Luc tumor cells ( $1 \times 10^6$  cells per mouse) via IV injection. On day 32, all mice were euthanized and the lungs were collected and observed by IVIS imaging system.

#### ***Anti-tumor effects of TAgD-TVac<sub>LLC</sub> in an orthotopic lung cancer mouse model***

The female C57BL/6 mice (Charles River) at 6~8-week old and luciferase stably expressed Lewis lung carcinoma (LLC-Luc) cells were used in this study. The C57BL/6 mice

were subjected to isoflurane anesthesia in an anesthesia box, and were then maintained in an anesthesia mask with the same flow rate of isoflurane on a heating pad warmed (37 °C). Expose the costal layer by removing the hair below the left scapula and disinfect the left costal area of the mice with 70% ethanol. 50  $\mu$ L of the prepared LLC-Luc cell suspension ( $0.5 \times 10^6$  per mouse) was aspirated into an insulin syringe. Insert the needle between the third and fourth costa through the chest wall and carefully pierce into the left lung. Inject the cells slowly at a depth of approximately 3-5 mm while keeping the syringe upright. Six days later, the establishment of the orthotopic lung cancer mouse model was verified by the IVIS imaging system. Then, the mice were randomly divided into four groups ( $n = 6$ ). Tumor-bearing mice received three doses of TAgD-TVac<sub>LLC</sub> (protein, 20  $\mu$ g per dose; LNP, 200  $\mu$ g per dose; SC vaccination at the tail base). Anti-PD-1 antibodies (aPD-1, 200  $\mu$ g per dose; three doses) were administrated by intraperitoneal (IP) injection. The inhibiting effect of single administration of TAgD-TVac<sub>LLC</sub> was also evaluated. Single administration of aPD-1 was used as the comparison. Tumor growth was monitored by measuring the *in vivo* bioluminescence using the IVIS imaging system (every three days). Since LLC-Luc tumors are expected to develop in the lungs, we do not expect any externally visible tumor masses, so we rely on body weight to assess tumor progression. The mice were euthanized when they exhibited >15% loss of body weight.

Lymph nodes and spleens were collected for the preparation of single-cell suspensions. The immune activation in lymph nodes and spleen was analyzed by staining the cells using fluorescent conjugated antibodies, including PE-anti-mouse-CD11C antibody, FITC-anti-mouse-CD80 antibody, APC-anti-mouse CD86 antibody, APC-anti-mouse CD3 antibody, PE-anti-mouse CD8 antibody, APC-Cy7-anti-mouse CD62L antibody, and FITC-anti-mouse CD44 antibody.

LLC-Luc tumor-bearing mice that have received aPD-1 + TAgD-TVac<sub>LLC</sub> combination therapy were rechallenged with LLC-Luc (left flank) and non-cognate B16F10 tumor cells (right flank) via SC injection on day 40 to mimic tumor recurrence ( $n = 5$ ). LLC-Luc cells ( $1 \times 10^6$  per mouse) were subcutaneously injected at the left flank of C57BL/6 mice, and B16F10 tumor cells ( $1 \times 10^6$  per mouse) were inoculated in the opposite flank of the LLC-Luc tumors. A control group (normal mice,  $n = 5$ ) was also treated with LLC-Luc and B16F10 tumor cells by SC injection. The tumor volume (V) was calculated according to the formula:  $V = L \times W^2/2$ . All mice were euthanized on day 55 and the tumors were collected for photograph.

***Anti-tumor effects of TAgD-TVac<sub>ID8</sub> in an inoperable ovarian cancer mouse model***

The female C57BL/6 mice (Charles River) at 6~8-week old and luciferase stably expressed ovarian (ID8-Luc) tumor cells were used in this study. ID8-Luc ( $1 \times 10^7$  per mouse) tumor cells were injected intraperitoneally into C57BL/6 mice for the establishment for inoperable ovarian cancer model. Seven days later, the establishment of the inoperable ovarian cancer mouse model was verified by the IVIS imaging system. Then, the mice were randomly divided into four groups ( $n = 6$ ). Tumor-bearing mice received three doses of TAgD-TVac<sub>ID8</sub> (protein, 20 µg per dose; LNP, 200 µg per dose; SC vaccination at the tail base). Anti-PD-1 antibodies (aPD-1, 200 µg per dose; three doses) were administrated by intraperitoneal (IP) injection. The anti-tumor effect of single administration of aPD-1 or TAgD-TVac<sub>ID8</sub> was also evaluated. Tumor growth was monitored by measuring the *in vivo* bioluminescence using the IVIS imaging system (every five days). Following institutional guidelines, mice were euthanized when they developed ascites and had a weight increase >30%.

Mice were bled on day 10. The levels of pro-inflammatory cytokines (including M-CSF, IFN- $\gamma$ , and IL-6) and pro-inflammatory chemokines (including MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) in the blood were measured with ELISA kits according to the manufacturer's instructions. When one of the tumor-bearing mice in PBS group reached humane endpoint criteria (weight increase >30%), all mice were euthanized to evaluate the ascites volume.

***Statistical Analysis***

Data were expressed as mean  $\pm$  SD. All data were analyzed using Graphpad Prism (8.0) software.

**INCORPORATION BY REFERENCE**

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

**EQUIVALENTS**

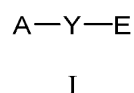
While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The

full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

## CLAIMS

We claim:

1. A compound having a structure represented by formula I or a pharmaceutically acceptable salt thereof:



wherein,

A is an antigen;

E is an active agent (e.g., a drug); and

Y is a linker which covalently links A to E and comprises an alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene, arylene, or heteroarylene, or a combination thereof, wherein the linker comprises 1-100 carbon atoms, preferably 10-50 carbon atoms.

2. The compound of claim 1, wherein the linker is a heteroalkylene comprising 5-50 moieties selected from -CH<sub>2</sub>-, -C(=O)-, -C(=O)-NR<sup>1</sup>-, -NR<sup>1</sup>-C(=O)-, -C(=O)-O-, -O-C(=O)-, -NR<sup>1</sup>-C(=O)-NR<sup>2</sup>-, -O-C(=O)-NR<sup>1</sup>-, -NR<sup>1</sup>-C(=O)-O-, -O-, and -S-; wherein each R<sup>1</sup> and R<sup>2</sup> is independently selected from H and alkyl.

3. The compound of claim 1 or 2, wherein the linker is a heteroalkylene comprising 5-50 moieties selected from -CH<sub>2</sub>- and -O-.

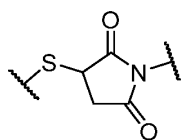
4. The compound of any one of claims 1-3, wherein the linker comprises ethylene glycol.

5. The compound of claim 4, wherein the linker comprises 1-10 ethylene glycol moieties (e.g., 2, 4, 6, or 8 ethylene glycol moieties).

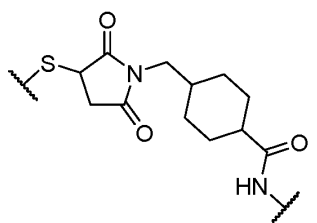
6. The compound of claim 1, wherein the linker is an alkylene comprising 5-50 methylene moieties.

7. The compound of claim 6, wherein the linker further comprises a moiety selected from  
 $-C(=O)-$ ,  $-C(=O)-NR^2-$ ,  $-NR^2-C(=O)-$ ,  $-C(=O)-O-$ ,  $-O-C(=O)-$ ,  $-NR^2-C(=O)-NR^3-$ ,  $-O-C(=O)-NR^2-$ ,  $-NR^2-C(=O)-O-$ ,  $-O-$ , and  $-S-$ ; wherein each  $R^2$  and  $R^3$  is independently selected from H and alkyl.
8. The compound of any one of claims 2-7, wherein the linker further comprises 1-5  $-C(=O)-$ ,  $-C(=O)-NR^2-$  or  $-NR^2-C(=O)-$  moieties (e.g., 1, 2, or 3 moieties); wherein each  $R^2$  and  $R^3$  is independently selected from H and alkyl.
9. The compound of any one of claims 2-8, wherein at least one  $-C(=O)-$  moiety is adjacent to E.

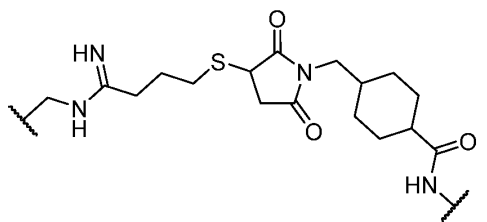
10. The compound of any one of claims 2-9, wherein the linker further comprises



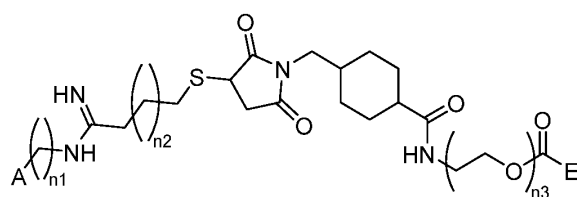
11. The compound of any one of claims 2-9, wherein the linker further comprises



12. The compound of any one of claims 2-9, wherein the linker further comprises



13. The compound of any one of claims 1-12, wherein the compound has a structure represented by formula Ia or a pharmaceutically acceptable salt thereof:



Ia

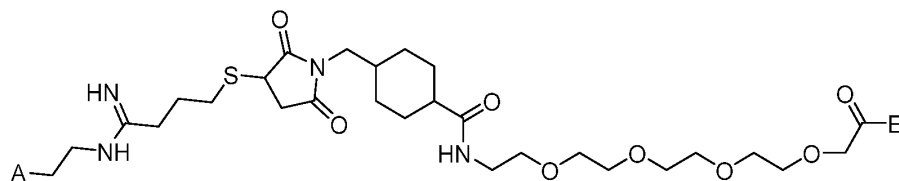
wherein

n1 and n2 are each independently selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,

16, 17, 18, 19, or 20; and

n3 is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

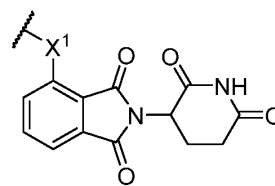
14. The compound of any one of claims 1-13, wherein the compound has a structure represented by formula Ib or a pharmaceutically acceptable salt thereof:



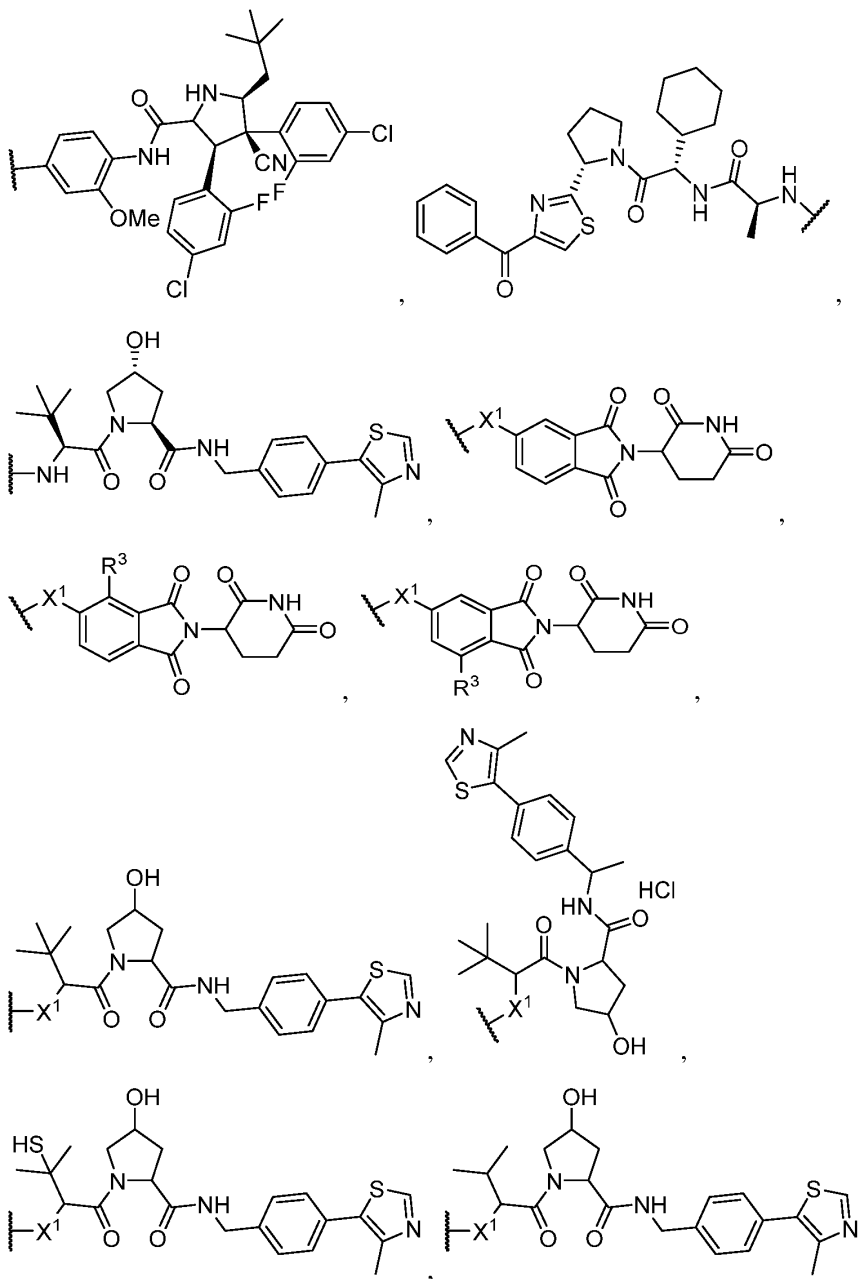
Ib.

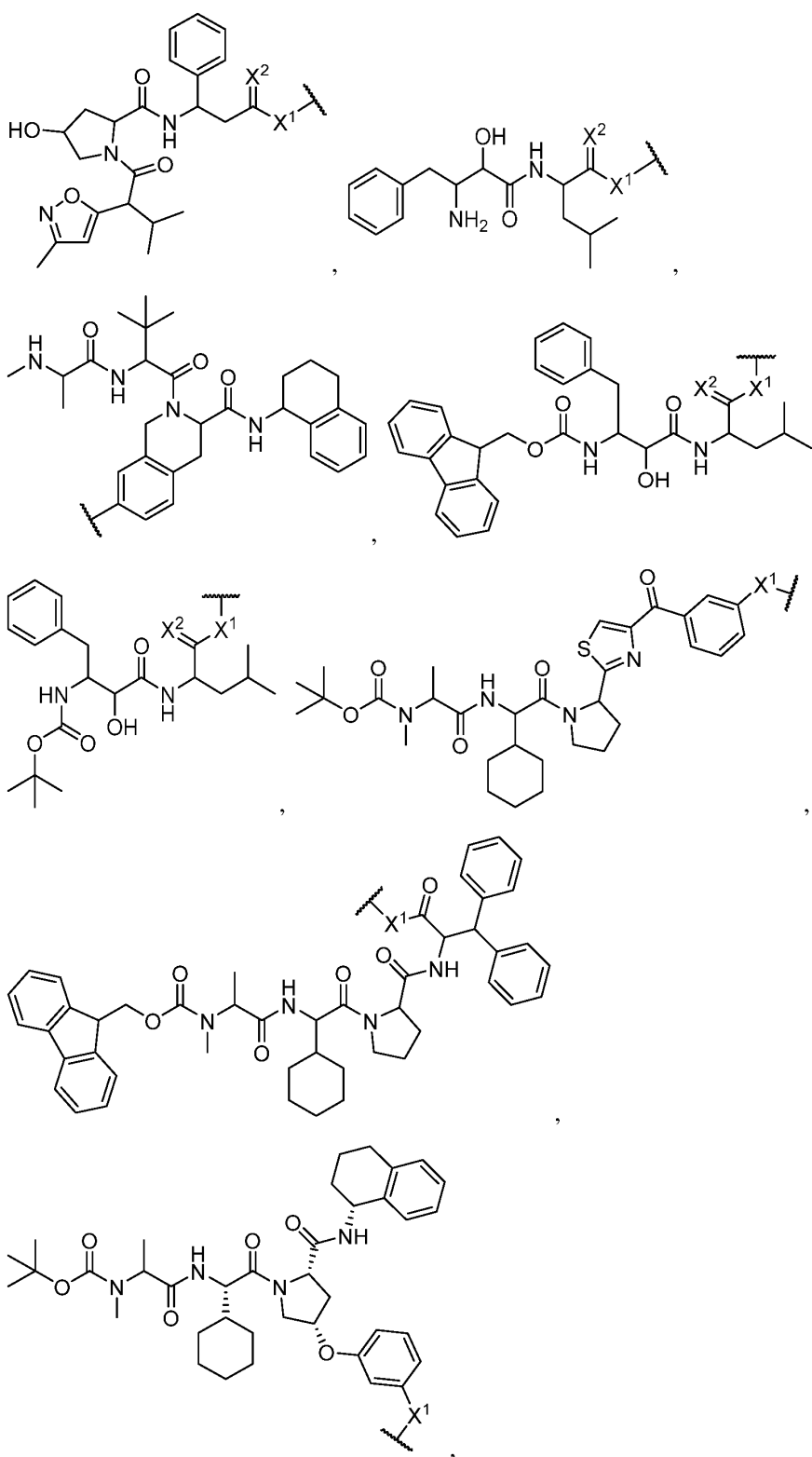
15. The compound of any one of claims 1-14, wherein E is a ligand for a ligase (*e.g.*, an E3 ligase).

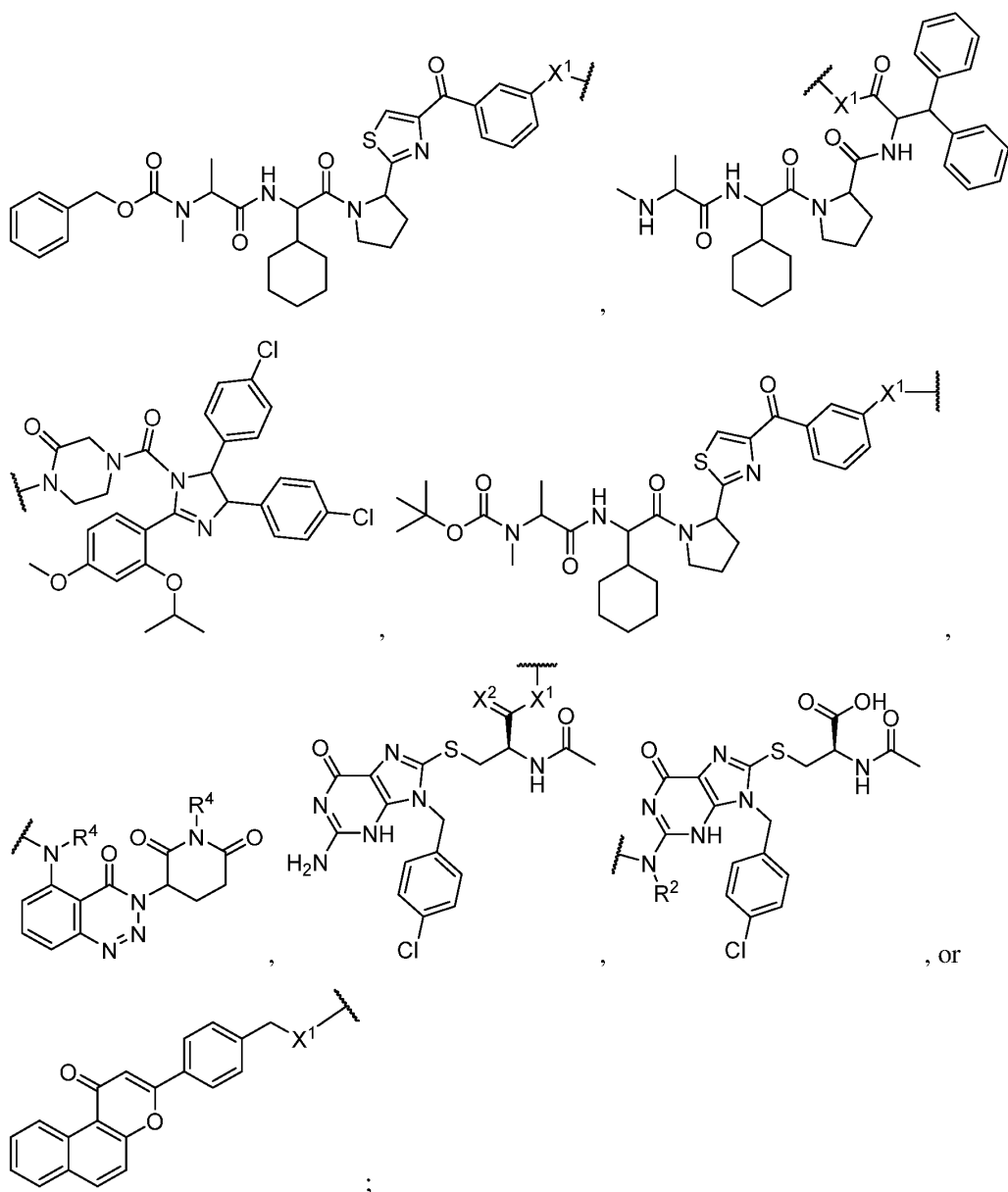
16. The compound of any one of claims 1-14, wherein E is a ligand for a von Hippel-Lindau (VHL), cellular inhibitor of apoptosis (cIAP), cereblon (CRBN), or murine double minute 2 (MDM2).



17. The compound of any one of claims 1-16, wherein E is



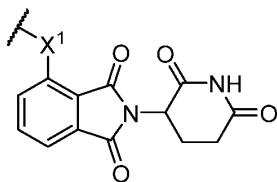




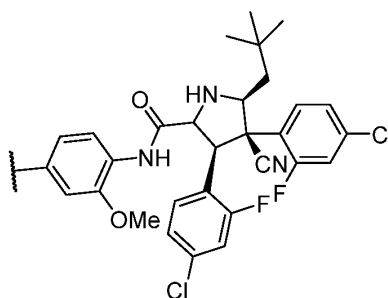
each  $X^1$  and  $X^2$  is independently selected from  $O$ ,  $NR^4$ , and  $S$ ;

each R<sup>3</sup> is independently selected from halo (e.g., fluoro or bromo); and

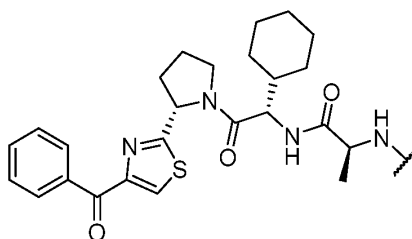
each R<sup>4</sup> is independently H, alkyl, or aralkyl.



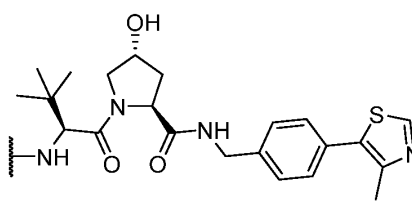
18. The compound of claim 17, wherein E is



19. The compound of claim 17, wherein E is

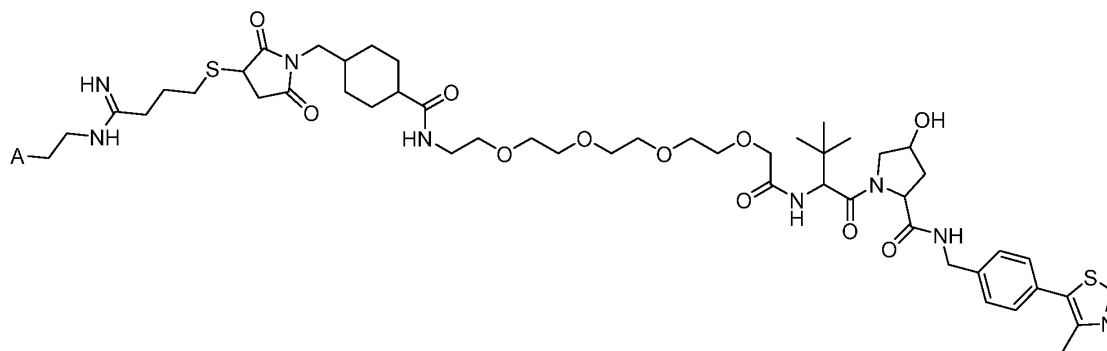


20. The compound of claim 17, wherein E is



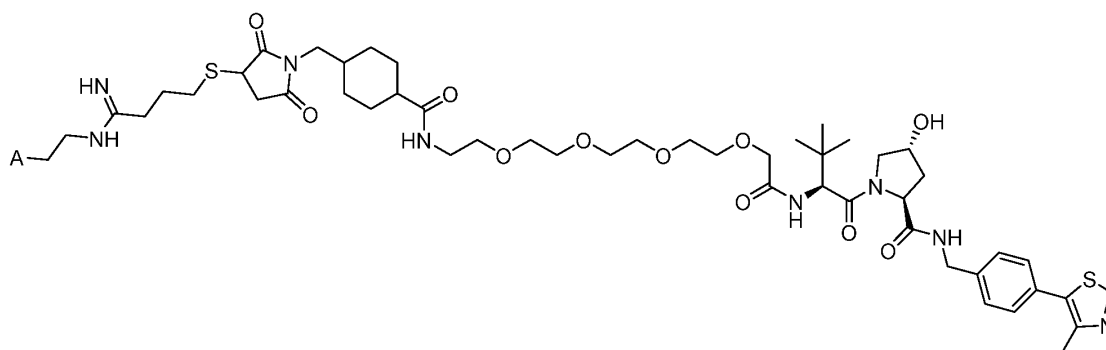
21. The compound of claim 17, wherein E is

22. The compound of claim 1, wherein the compound has a structure represented by formula Ic or a pharmaceutically acceptable salt thereof:



Ic.

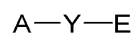
23. The compound of claim 1, wherein the compound has a structure represented by formula Id or a pharmaceutically acceptable salt thereof:



Id.

24. The compound of any one of claims 1-23, wherein A is an T antigen.
25. The compound of any one of claims 1-24, wherein the antigen is from a tumor (e.g., from tumor cells).
26. The compound of any one of claims 1-24, wherein the antigen is from an infection pathogen (e.g., a bacterium or virus).
27. The compound of any one of claims 1-24, wherein the antigen is from prostate cancer tumor cells, liver tumor cancer cells, colorectal tumor cancer cells, ovarian tumor cancer cells, pancreatic tumor cancer cells, ovarian tumor cancer cells, breast tumor cancer cells, melanoma tumor cancer cells, or bladder tumor cancer cells.
28. The compound of any one of claims 1-24, wherein the antigen is prostate antigen (PSA), alphafetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA-125), mucin-1 (MUC-1), melanoma-associated antigen (MAGE), or New York esophageal squamous cell carcinoma 1 (NY-ESO-1).
29. The compound of any one of claims 1-24, wherein A is a Simian Vacuolating Virus 40 T antigen (SV40 large TAg).
30. A nanoparticle, comprising a tumor antigen covalently linked to an active agent; and a plurality of lipids or lipidoids.

31. The nanoparticle of claim 30, wherein the tumor antigen covalently linked to an active agent has a structure represented by formula I:



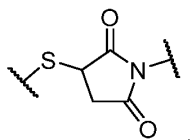
I

A is the tumor antigen;

E is the active agent (e.g., a drug); and

Y is a linker which covalently links A to E and comprises an alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene, arylene, or heteroarylene, or a combination thereof, wherein L comprises 1-100 carbon atoms, preferably 10-50 carbon atoms.

32. The nanoparticle of claim 30 or 41, wherein the tumor antigen is covalently linked to



the active agent via

33. The nanoparticle of claim 30, wherein the active agent is a compound of any one of claims 1-29.

34. The nanoparticle of any one of claims 30-33, wherein the nanoparticle comprises a plurality of lipidoids.

35. The nanoparticle of any one of claims 30-33, wherein the nanoparticle comprises a plurality of lipids.

36. The nanoparticle of any one of claims 30-33, wherein the nanoparticle comprises a plurality of lipids and lipidoids.

37. The nanoparticle of any one of claims 30-36, wherein the lipids and lipidoids are selected from the group consisting of 113-O12B, cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG<sub>2k</sub>), MC-3, SM-102, ALC0315, L88, and 93-O17S or combination of any of the foregoing.

38. The nanoparticle of any one of claims 27-33, wherein the lipids and lipidoids are selected from the group consisting of 113-O12B, cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG<sub>2k</sub>).
39. A pharmaceutical composition, comprising the nanoparticle of any one of claims 30-38; and a pharmaceutically acceptable excipient.
40. A method of treating cancer, comprising administering to a subject in need thereof a therapeutically effective amount of the compound of any one of claims 1-29, or a pharmaceutically acceptable salt thereof, or the nanoparticle of any one of claims 30-38.
41. The method of claim 40, wherein the cancer is melanoma, breast cancer, ovarian cancer, spleen cancer, or lung cancer.
42. The method of claim 40 or 41, wherein the method further comprises administering an immune checkpoint inhibitor (e.g., a PD-1 inhibitor).
43. A method of treating an infectious disease, comprising administering to a subject in need thereof a therapeutically effective amount of the compound of any one of claims 1-29, or a pharmaceutically acceptable salt thereof, or the nanoparticle of any one of claims 30-38.
44. The method of claim 43, wherein the infectious disease pathogen is a bacterium.
45. The method of claim 43, wherein the infectious disease pathogen is a virus.