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(54) Title: COMPOSITIONS AND METHODS FOR TREATMENT OF DISTENTABLE TISSUES

(57) Abstract: Provided herein are compositions and methods for treating cancer in a distentable tissue, for example, a bladder, a uterus, peritoneum, omentum, or eye. Useful compositions include DT-EGF fusion proteins as described herein.



WO 2020/219599 A1

COMPOSITIONS AND METHODS FOR TREATMENT OF DISTENTABLE TISSUES

Related Applications

[0001] This application claims the benefit of U.S. Provisional application No. 62/837,533 titled “Compositions and Methods for Treatment of Distentable Tissues” filed April 23, 2019 which is incorporated herein by reference.

Sequence Listing

[0002] An official copy of the sequence listing is submitted concurrently with the specification electronically via EFS-Web as an ASCII formatted sequence listing with a file name of “10037.001WO1_ST25”, a creation date of April 22, 2020, and a size of about 18.8 kilobytes. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

Technical Field

[0003] The present disclosure relates to new therapeutics and methods for treatment of distentable tissues, particularly, treatment of cancer in distentable tissues.

Background

[0004] Drugs have specificity for disease cells or are designed to specifically target elements of the diseased cells. All drugs, regardless of mechanism, must bind and/or be taken up by their respective target cell of interest. Unfortunately, in the practical settings of disease treatment, drugs rarely if ever bind all targets or achieve uniform targeting of the whole population of diseased cells. This is one cause of incomplete response and recurrence of disease.

Brief Summary

[0005] Provided herein are pharmaceutical compositions and methods for treating cancer in distentable tissues.

[0006] In some embodiments, a pharmaceutical composition comprising a diphtheria toxin-epidermal growth factor (DT-EGF) fusion protein of structure A-X-Y-Z, wherein: A is 0-5 amino acid residue N-terminal addition to the front of the diphtheria toxin sequence; X is a diphtheria toxin

fragment or mutated fragment that maintains catalytic activity; Y is sequence of amino acids from 0 – 20 aa in length that connects the carboxy terminus of X to the amino terminus of Z; and Z is an epidermal growth factor sequence or mutant of that sequence that maintains or improves binding affinity for epidermal growth factor receptor.

[0007] In some aspects, the composition has a pH of at least about 7.0, or a pH of at least about 7.4, or a pH of at least about 8.0.

[0008] In some embodiments, the composition is formulated with: 10 mM PO₄ and 150 mM NaCl at pH 8.0. In some aspects, the composition is formulated with 100 mM glucose.

[0009] In some aspects, A is a single alanine.

[0010] In some aspects, X is (glycosylation sites removed)

gaddvvdss ksfvmenfas yhgtpgyvd siqkgiqkpk sgtqgnyddd
 wkgfystdnk ydaagysvdn enplsgkagg vkvtypglt kvlalkvdna etikkelgls
 lteplmeqvg teefikrfgd gasrvvslp faegssvey innweqakal sveleinfet
 rgkrgqdamy eymaqacagn rvrrsvgssl scinldwdvi rdktktkies lkehgpi knk
 msespaktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya awavnvaqvi
 dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaiplvge
 lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf (SEQ ID NO: 1)

[0011] In some aspects, X is (wild type sequence)

gaddvvdss ksfvmenfss yhgtpgyvd siqkgiqkpk sgtqgnyddd
 wkgfystdnk ydaagysvdn enplsgkagg vkvtypglt kvlalkvdna etikkelgls
 lteplmeqvg teefikrfgd gasrvvslp faegssvey innweqakal sveleinfet
 rgkrgqdamy eymaqacagn rvrrsvgssl scinldwdvi rdktktkies lkehgpi knk
 msespnktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya awavnvaqvi
 dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaiplvge lvdigfaayn
 fvesiinlfq vvhnsynrpa yspghktqpf (SEQ ID NO: 2)

[0012] In some aspects, X is a modified sequence that maintains catalytic activity while also evading immune response.

[0013] In some aspects, Y is ha, pw, lp, aa, or gg.

[0014] In some aspects, Y is:

- a. A short sequence of 0 – 20 amino acids designed to be targeted by lysosomal or intracellular proteases

- b. A short sequence of 0 – 20 amino acids designed to minimize negative or inhibitory interactions of X and Z sequences.

[0015] In some aspects, Z is wild type human EGF sequence.

[0016] In some aspects, Z is a mutant that increases affinity for the EGF receptor. In some aspects, Z is:

wnsYsecp PsYdgyclhd gvcRyieald Syacncvvgv Agercqyrdl RwwGRr (SEQ ID NO: 3).

[0017] In some aspects, XYZ is:

gaddvvdss ksfvmenfas yhgkpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk
ydaagysvsn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd
gasrvvlslp faegsssvy innweqakal sveleinfet rgkrqdamy eymaqacagn
rvrrsvgssl scinldwdvi rdktktkies lkehgpi knk msespaktvs eekakqylee fhqtalehpe
lsselktvtgt npvfaganya awavnvaqvi dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv
aqsalsslm vaqaiplyge lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsYsecp
PsYdgyclhd gvcRyieald Syacncvvgv Agercqyrdl RwwGRr (SEQ ID NO: 4)

[0018] In some aspects, AXYZ is:

agaddvvdss ksfvmenfas yhgkpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk
ydaagysvsn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd
gasrvvlslp faegsssvy innweqakal sveleinfet rgkrqdamy eymaqacagn
rvrrsvgssl scinldwdvi rdktktkies lkehgpi knk msespaktvs eekakqylee fhqtalehpe
lsselktvtgt npvfaganya awavnvaqvi dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv
aqsalsslm vaqaiplyge lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsdsecp
lshdgyclhd gvcmyieald kyacncvvgv igercqyrdl kwwelr (SEQ ID NO: 5) (A-dmDT390-EGF)

[0019] In some aspects, AXYZ is:

agaddvvdss ksfvmenfas yhgkpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk ydaagysvsn
enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd gasrvvlslp
faegsssvy innweqakal sveleinfet rgkrqdamy eymaqacagn rvrrsvgssl scinldwdvi
rdktktkies lkehgpi knk msespa ktvs eekakqylee fhqtalehpe lsselktvtgt npvfaganya
awavnvaqvi dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaiplyge
lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsYsecp PsYdgyclhd gvcRyieald
Syacncvvgv Agercqyrdl RwwGRr (SEQ ID NO: 6)

[0020] The above compositions can be used in the methods described below and throughout the description. In some aspects, provided herein is a pharmaceutical composition for use in the manufacture of a medicament for use in the methods disclosed herein. In some aspects, the pharmaceutical composition, i.e. the targeted drug treatment, comprises SEQ ID NO: 5 or SEQ ID NO: 6. In some aspects, the targeted drug treatment is SEQ ID NO: 5. In some aspects, the targeted drug treatment is SEQ ID NO: 6.

[0021] Provided herein is a method to treat cancer in a distensible organ with an instilled volume that maximizes exposure of cellular surface targets to a targeted drug treatment. In some aspects, the targeted drug treatment is SEQ ID NO: 5. In some aspects, the targeted drug treatment is SEQ ID NO: 6.

[0022] Provided herein is a method to treat cancer in a distensible organ with an instilled volume comprised of a formulation and set of conditions that maximizes exposure of cellular surface targets to a targeted drug treatment. In some aspects, the targeted drug treatment is SEQ ID NO: 5. In some aspects, the targeted drug treatment is SEQ ID NO: 6.

[0023] Provided herein is a method to treat cancer in a distensible organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that maximizes exposure of cellular surface targets to a targeted drug treatment. In some aspects, the targeted drug treatment is SEQ ID NO: 5. In some aspects, the targeted drug treatment is SEQ ID NO: 6.

[0024] Provided herein is a method to treat cancer in a distensible organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that removes constituents that inhibit binding and exposure of cellular surface targets to a targeted drug treatment. In some aspects, the targeted drug treatment is SEQ ID NO: 5. In some aspects, the targeted drug treatment is SEQ ID NO: 6.

[0025] Provided herein is a method to treat cancer in a distensible organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that maximizes treatment efficacy by mechanisms other than improved binding of drug to target. An example of a pre-dosing treatment would be as that shown in Figure 1 – e.g. instill up to 500 mL of sterile isotonic saline with 10 mM Citrate pH 6.0, 37°C, 0.01% sodium dodecylsulfate and hold for 15 minutes, subsequently empty bladder and flush and empty up to 3x with 500 mL sterile isotonic saline. This causes a breakdown and removal of glycocalyx and other binding inhibitors.

[0026] Also provided herein is a method to treat cancer in a distensible organ with an instilled volume comprised of a formulation and set of conditions in a post-dosing treatment that maximizes treatment efficacy by mechanisms other than improved binding of drug to target. An example of a post-dosing treatment includes instilling up to 500 mL and holding for up to 1 hour a solution of 0.25% Acetic Acid at pH 4.5 and 40 °C, subsequently emptying the bladder and flushing and emptying 3x with up to 500 mL sterile isotonic saline. This enables drug release from endosome facilitated by acid and the heat induced increase in chaperone proteins – increased cytoplasmic drug concentration and activity.

[0027] The cancer can be in the bladder, the pleura, the uterus, the peritoneum, the eye, or the omentum. In some aspects, the cancer is in the bladder.

[0028] In some aspects, the pharmaceutical composition is a protein-toxin fusion of Diphtheria toxin to Epidermal Growth factor, a protein-toxin fusion of Pseudomonas Exotoxin A to EpCAM, or the protein-toxin fusion of IL-2 with diphtheria toxin known as Denileukin diftitox or ONTAK.

Description of the Figures

[0029] Figure 1 provides a schematic demonstrating the treatment of cancer such as bladder cancer by distension.

[0030] Figure 2 demonstrates binding measurements and analyses of two DTEGF fusion-toxins to EGF by Surface Plasmon Resonance (SPR). SPR sensorgram (black lines) and the results from non-linear least squares regression analysis of the data (red lines) are shown. The graphs are the sensorgrams and the global fit to a 1:1 binding model for duplicate injections of six analyte concentrations ranging between 300nM to 1.23 nM against captured rhu EGFR (ligand).

[0031] Figure 3 shows A-dmDT390-EGF and A-dmDT390-EGF-Kd+ / rhuEGFR interactions, including k_a , k_d , R_{max} , and K_D , as buffer conditions are adjusted.

[0032] Figure 4 demonstrates cytotoxicity of A-dmDT390-EGF (BO5) and A-dmDT390-EGF-Kd+ (BO1) in HTB9Luc cells after 15 minutes of treatment.

[0033] Figure 5 depicts improved HTB9 cytotoxicity of A-dmDT390-EGF achieved by including 10 mM or 100 mM glucose in the treatment buffer.

Detailed Description

[0034] Provided herein are novel compositions of fusion proteins composed of natural occurring and modified amino acid sequences of Diphtheria Toxin (DT) and Epidermal Growth Factor (EGF) and the amino acid sequence that joins them. DT-EGF fusion proteins have been developed as potential therapeutics to treat cancer and disease driven by cells that overexpress Epidermal Growth Factor Receptor (EGFR). The methods and compositions disclosed herein can be applied to any DT-EGF fusion protein to minimize or eliminate immune inactivation and to improve its efficacy by enhancing the activities of the respective drug moieties: DT's catalytic activity driving ribosomal inactivation and facilitating endosomal release, EGF's binding of receptors and cellular uptake, and linking amino acid sequences ability to maximize the independence of the DT and EGF activities. The linker may include an amino acid sequence that provides susceptibility to intracellular protease to enable release of the DT from the EGF moiety once inside the target cell. The methods and compositions disclosed herein provide a novel means of avoiding immune inactivation of DT-EGF, enhancing specific binding to target receptors on diseased cells, enhancing targeted uptake and intracellular release, enhancing intracellular toxicity of the DT moiety, and minimizing interference of the DT with the EGF moiety functions (and vice versa).

[0035] To further extend the efficacy of fusion protein drugs like DT-EGF, and any drug that does not depend upon passive diffusion for activity, is provided herein a novel method for treatment of diseases of distensible tissues, where instillation or injection into a distensible tissue such as bladder, pleura, uterus, peritoneum, omentum, or eye is the route of administration; a distensible tissue is any tissue that can hold a defined volume of fluid for a minimum of 5 minutes and may or may not contain transitional epithelium. The method can be applied to any therapeutic treatment that requires active or facilitated transport across the target cells plasma membrane, and/or docks to, and/or chemically alters, and/or binds to biologic targets on the plasma membrane of the targeted cells. Examples include but are not limited to small molecules (that use facilitated uptake mechanisms), biologics—fusion-proteins, immunotoxins, antibodies, gene therapies and oncolytic virus or vaccines. The method is not useful for drugs that use passive diffusion to enter the target cells. This method enables treatment, or enhances efficacy of existing treatments through pre-dosing, dosing and post-dosing formulations, conditions and tissue conditioning. The pre-dosing and dosing formulations and conditions maximize drug-target binding interactions. The method includes distention of the target organ to mechanically stretch the tissue and thereby maximize exposure of

targets on the cell membranes to targeted therapies. Post-dosing formulations and conditions facilitate post-binding events, such as intracellular uptake, release of drug from endosomes or lysosomes, or chaperone functions that enhance efficacy.

[0036] The present methods relate to the use of DT-EGF fusion proteins and other drugs that must interact with defined receptor structures exposed on surface of target cells—such as EGF receptors, tumor antigen or biomarkers—used in a manner that enables (i) maximum binding of the drug to its target, (ii) maximum binding specificity and uptake of drug by target cells, (iii), post-binding events including cellular uptake and release into the cytoplasm or other intracellular organelle, (iv) minimum time required to approach binding saturation (iv) optimal time and conditions to maximize efficacy (v), minimum non-specific off-target drug uptake and (vi) subsequent minimization of side effects and undesired toxicity. This provides a means of treating disease involving the tissues in distentable organs and specifically of utilizing DT-EGF drugs for treating disease associated with EGFR and/or related EGFR receptor family expression.

[0037] DT-EGF has been considered for development as a potential anticancer therapy for over twenty years¹⁻¹¹. In the few DT-EGF fusion proteins that have been constructed and tested, the naturally occurring DT sequence has had its binding domain replaced with the naturally occurring EGF sequence with 0-5 intervening amino acids acting as a linker. The impact of the linker of EGF domain upon the DT toxicity has not been assessed; however, it has been reported that the addition of the linker and DT sequence to the N-terminus of the EGF sequence has reduced its binding by up to 30-fold and its subsequent signal transduction.

[0038] The EGF-EGFR interactions have been well studied. In purified systems a number of mutants in the EGF sequence have demonstrated significant decreases in binding EGFR, while others have demonstrated increased binding and/or increased association rates¹². As noted above, the addition of the DT sequence has had a negative impact upon receptor binding by the EGF moiety.

[0039] While DT driven toxicity of DT-EGF constructs has been demonstrated in vitro and in vivo, the magnitude of the impact of the linker and EGF moiety upon the DT induced toxicity is unknown.

[0040] In some aspects, a linker sequence that is susceptible to lysosomal and or intracellular proteases that enable release of the EGF moiety will increase the toxicity of the DT moiety and thereby increase the efficacy of the DT-EGF drug construct.

[0041] Many people have received vaccination for Diphtheria and therefore possess varying levels of inactivating antibodies that can decrease the efficacy of DT-EGF constructs. By incorporating mutations in the sequence that are reported to decrease DT immunogenicity¹³, improvement in DT-EGF efficacy can be achieved.

[0042] Provided herein are combinations of the three elements mentioned above, (i) EGF mutants to improve binding, (ii) linker sequence to minimize DT, EGF cross-inhibition (e.g. as in the case where linking caused a 20-fold reduction in the affinity of the EGF moiety to EGFR) or enable intracellular release of DT (e.g. a cleavable linker that releases the DT moiety once inside the target cell), and (iii) mutations that de-immunize the DT moiety.

[0043] Minor changes, including single amino acid residue substitutions, can have profound effects on activity in unpredictable ways; thus, the method outlined above to improve DT-EGF construct activity and efficacy will enable reasonable predictions but will ultimately require empirical validation. Several mutations in the EGF moiety were attempted, and most failed to exhibit improved EGFR binding.

[0044] The following sequence is considered the standard that contains essentially wild type DT and EGF sequences joined by a sequence of amino acids that is amenable to in vivo activity but with a known 30-fold reduction in EGF to EGFR binding affinity: the amino acid sequence of protein 1 (A-dmDT390-EGF), a fusion of a truncated Diphtheria toxin with Epidermal Growth Factor.

[0045] Information on the sequences include: 1, Additional alanine residue; 2~391, dmDT390; 19, S to A (mutation for removal of N-glycosylation site); 236, N to A (mutation for removal of N-glycosylation site); 392~393, amino acid sequence for NcoI site; 394~446: EGF
 agaddvvdss ksfvmenfas yhgkpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk ydaagysvsn
 enplsgkagg vvkvtypglt kvllalkvdna etikkelgls lteplmeqvg teefikrfgd gasrvvlslp
 faegssvey innweqakal sveleinfet rgkrgqdamy eymaqacagn rvrrsvgssl scinldwdvi
 rdktktkies lkehgpi knk msespa ktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya
 awavnvaqvi dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaiplyge
 lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsdsecp lshdgyclhd gvcmyieald
 kyacncvgy igercqyrdl kwwelr (SEQ ID NO: 5)

[0046] Too often drugs benefit only a small fraction of patients with relapse common in responders. The current invention aims to increase the fraction of responders and decrease the

frequency of relapse through a method that enhances existing drug efficacy or enabling optimal efficacy in novel candidate drugs.

[0047] Drugs have specificity for disease cells or are designed to specifically target elements of the diseased cells. All drugs, regardless of mechanism, must bind and/or be taken up by their respective target cell of interest. Unfortunately, in the practical settings of disease treatment, drugs rarely if ever bind all targets or achieve uniform targeting of the whole population of diseased cells. This is one cause of incomplete response and recurrence of disease. In one aspect, use of pre-dosing and dosing conditions including benign distention of the target tissue to achieve maximum target binding enhances therapeutic benefit. In another aspect, post-dosing instillation of a formulation under conditions designed to enhance post-drug binding events improves drug activity and efficacy.

[0048] The potential to hold volume for a set period of time within an organ or tissue—a ‘distentable tissue’—is currently used to treat disease. However, intentional distending of an organ or tissue, such as a bladder, to increase its drug uptake has not been reported or proposed. Intravesicular treatments for bladder cancer are presented as examples of the current approaches being used and to contrast the differences and benefits enabled by the current invention; the concept can be extended to any distentable tissue. A healthy adult will typically empty their bladder when it has filled to between 250 – 500 mL; however, the bladder may hold up to 1000 mL without damage. The shape of the bladder and the interior surface area obviously change greatly during filling and transition from empty to distended (250mL – 1000mL). The urothelium lining the interior of the bladder is transitional epithelium, so named, because it can transition from a compacted structure to an extended structure while maintaining its integrity. The layers of transitional epithelial cells of the urothelium undergo extreme shape changes as the bladder fills and ultimately the superficial lining cells appear irregular and squamous as they are stretched and expose their greatest surface area—see Figure 1. It is this mechanism that the present disclosure exploits to enable the requisite interactions of drug with cell surface structures, which surprisingly and unexpectedly maximizes drug activity.

[0049] With current therapies for bladder cancer and those in clinical testing, volumes of 20-75 mL are intravesicularly delivered into an empty bladder and then held within the bladder for 2 hours; this is true for small molecules therapeutics (e.g. Gemcytabine, apaziquone), biologics proteins (e.g. VB4-845) or nucleic acid based gene therapies (e.g. BC819) or oncolytic virus (e.g. CG0700, rAd-IFN).

[0050] A pre-dosing wash of the bladder contents can improve the ability of a drug to bind targets on the bladder lining—urothelium. One existing approach demonstrating improved uptake of a potential therapeutic oncolytic virus is the disruption or removal of the glycocalyx. The glycocalyx is a glycoprotein layer between the uroepithelial cell layer and the contents of the bladder. Viral infection of the urothelium can be increased by flushing the bladder with a mild detergent solution designed to remove or disrupt the glycocalyx¹⁴. An example of this is the prewash used prior to instilled treatment with rAD-IFN virus—currently in clinical trials to treat bladder cancer. While the pre-wash condition used prior to CG0700-rAD-INF treatment may benefit other drugs with different uptake mechanisms, different prewash formulations and conditions improve binding and uptake of those different drugs. It has been demonstrated in a different system that disruption of the glycocalyx by treatment with neuraminidase facilitates HLA/anti-HLA-mediated cell attachment: a 3.6-fold increase of the two-dimensional binding constant was reported¹⁵. The goal of a prewash is to remove factors like the glycocalyx that would interfere with the binding and uptake of drug while simultaneously minimizing any damage or change to the urothelium that could be harmful; the balance between these two requires empirical determination, and the prewash provided herein is surprisingly effective.

[0051] A very small range (20-75 mL) of drug solution volumes are typically instilled in the bladder; no clinical investigation of efficacy as a function of volume has been presented. A study in mice reported no difference in virus uptake in the bladder urothelium between a 50 and 100 uL instilled volume; (it was not noted that both volumes are well below the >150 uL needed for pronounced bladder distention in mice). Distention has not previously been proposed to increased accessibility of binding sites for drugs or other binding interactions. The present disclosure provides that maximizing the surface area of the target tissue by distention enables access to the maximum number of target sites for drug entry into the cell. Moreover, in the case of bladder distention an added and surprising benefit is induction of reduced flow from the ureters and subsequent minimization of the changes to the binding buffer/solution instilled into the bladder.

[0052] The constituents of urine: water, urea, creatinine, proteins, hormones, inorganic salts and other organic compounds with a range in pH of about 5.5 – 7, negatively impact binding interactions between disease cells and the treatments designed to kill or modify them.

[0053] In some aspects, utilization of a dosing solution for instillation is provided, such that binding conditions are maximized and maintained for a sufficient time to approach saturation of binding sites.

[0054] Elements of instillation to be utilized include the following: time, volume, temperature, pH, viscosity, dielectric, ionic strength, monovalent ions, divalent ions, excipients that act as volume excluders, detergents, chaotropic agents, stabilizers. Colorization agents used to monitor and assess the degree of dilution caused by addition or urine during the treatment time.

[0055] The optimal dosing solution with best conditions and time required for ideal binding cannot be predetermined. The need to empirically determine—with purified systems, cell-based testing or in vivo models—optimal formulations and conditions is a function of the complexity of system. The optimal conditions reported for EGF binding to EGFR have demonstrated that even small changes in ligand proteins or their receptor sequences significantly and measurably change binding in ways that cannot be predicted (Cochran et al. US 8,865,864 B2). Relatively small changes in a sequence have significant and measurable differences in binding on- and off-rates and affinity. The N-terminal addition of long sequences, such as the diphtheria toxin used in the DT389EGF fusion-toxin¹⁶ can affect the binding to the EGFR. Essentially all biological interactions of interest (e.g., ligand-receptor, protein-protein interaction, small molecule-transport proteins and transmembrane transport of various compounds) have complex dependency on pH, temperature, concentration, viscosity, dielectric constant, ionic strength and solute concentration of most any type. This indicates the need to empirically determine the optimal binding conditions for any given change in sequence or environment.

[0056] Large changes such as attachment of antibody or toxin sequence elicits unknown changes in the requirements for the following conditions to demonstrate affinity of ligand for receptor: temperature, ligand concentration, monovalent and divalent concentration, chaotropic excipient concentration, need for prewash, pH, viscosity and binding time.

[0057] A report of the bladder-cancer cell killing ability of DT398EGF showed that 24-hour exposure of drug caused more cell death than a 2-hour pulse of the drug¹⁷. However, the minimal or optimal time required was not investigated in cell culture and thus has not been a parameter tested in the in-vivo setting. As noted above, convention for current oncology therapies is a 2-hour hold of an instilled drug formulation. While the time required for binding in a given formulation and under specified conditions in a clinical setting cannot be predicted, we can set boundaries to initiate

empirical optimization. Reports measuring binding rates in purified EGF/EGFR systems indicate complete binding on the minutes time scale, while the cell based system above indicates hours to days may be needed. As such, a time point between 10 minutes and 48 hours is contemplated herein to drive optimal efficacy. If more than 2-hours are needed, continuous irrigation is used. The flow rates into and out of the bladder, and the bladder volume can be maintained such that constant drug concentrations (within practical ranges and above the determined therapeutic threshold) and formulation conditions can remain optimal for the required amount of time using current clinical procedures used by those skilled in the art.

[0058] The above discussion emphasized the parameters which impact and allow improved efficacy of the binding events themselves. Pre-dosing, dosing and post-dosing conditions upon the target organ and target cells also impact the efficacy or reduction in toxicity of many treatments. Reports¹⁸ have demonstrated that the acidification of lysosomes is important to diphtheria toxin release into the cytoplasm; acidification of the cell media increases toxicity while blocking acidification reduces or prevents toxicity. Thus, pH is important to more than just binding. Reports¹⁹ have also demonstrated the heat shock proteins are important chaperone proteins that aid in the translocation of diphtheria toxin from endosomes into the cytoplasm. Thus, temperature - and its use to induce increased chaperone protein activity—may be used to increased toxicity through mechanisms unrelated to binding. Thus, exposure to altered temperatures and pH by the target organ and/or cells for time prior, during or post-dosing can have significant effects upon activity by protein-toxin fusion therapies and other therapies.

[0059] Provided herein are compositions and methods for treating disease that has overexpression of EFG-receptors on its cell surface by administering DT-EGF fusion proteins to a subject in need thereof. In one aspect, the disclosed methods may reduce the immune response caused by the sequences of DT by altering the amino acid is such a way that does not significantly affect DT-induced toxicity. In another aspect, the disclosed methods may increase the affinity of the EGF moiety of DT-EGF constructs for the EGF receptors. In another aspect, the disclosed methods may insert amino acids sequence between the DT and EGF moieties to minimize any antagonism—such as reduce EGFR binding or reduced DT-induced toxicity—or enable release of DT from the DT-EGF construct once inside the target cell.

[0060] In one embodiment the method combines the naturally occurring DT-sequence with the binding domain replaced with a short sequence of 0 – 20 amino acids to EGF that has been modified with any of the mutations reported to increase EGF-receptor association rate or affinity. An exemplary embodiment includes the amino acid sequence of protein (A-dmDT390-EGF-Kd+), a fusion of a truncated Diphtheria toxin with an enhanced binding Epidermal Growth Factor mutant.

[0061] Mutations to the XYZ sequence below are indicated with capital letters.

agaddvvdss ksfvmenfas yhgtpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk ydaagysvsn
 enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd gasrvvlslp
 faegsssvvey innweqakal sveleinfet rgkrqdamy eymaqacagn rvrrsvgssl scinldwdvi
 rdktktkies lkehgpi knk msessa ktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya
 awavnvaqvi dsetadnlek ttaalsilpg igsvmgia dg avhhnteeiv aqsialsslm vaqaipvlge
 lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsYsecp PsYdgyclhd gvcRyieald
 Syacncvgy Agercqyrdl RwwGRr (SEQ ID NO: 6)

[0062] In some aspects, Z is an EGF with modifications improving EGFR binding characteristics. For example, Z can be:

wnsYsecp PsYdgyclhd gvcRyieald Syacncvgy Agercqyrdl RwwGRr (SEQ ID NO: 3)

[0063] In one embodiment the method combines incorporation or reported sequence modifications that reduce immune recognition of DT-sequence with the binding domain replaced with a short sequence of 0 – 20 amino acids to EGF that retains the naturally occurring amino acid sequence.

[0064] In one embodiment the method combines incorporation or reported sequence modifications that reduce immune recognition of DT-sequence with the binding domain replaced with a short sequence of 0 – 20 amino acids to EGF that has been modified with any of the mutations reported to increase EGF-receptor association rate or affinity.

[0065] In one embodiment the method combines the naturally occurring DT-sequence with the binding domain replaced with a short sequence of 0 – 20 amino acids designed to be targeted by lysosomal or intracellular proteases - to EGF that has been modified with any of the mutations reported to increase EGF-receptor association rate or affinity.

[0066] In one embodiment, the method combines incorporation or reported sequence modifications that reduce immune recognition of DT-sequence with the binding domain replaced

with - a short sequence of 0 – 20 amino acids designed to be targeted by lysosomal or intracellular proteases - to EGF that retains the naturally occurring amino acid sequence.

[0067] In one embodiment the method combines incorporation or reported sequence modifications that reduce immune recognition of DT-sequence with the binding domain replaced with a short sequence of 0 – 20 amino acids designed to be targeted by lysosomal or intracellular proteases - to EGF that has been modified with any of the mutations reported to increase EGF-receptor association rate or affinity.

[0068] In one aspect, provided herein is a method to treat disease in a distentable organ in a subject, the method comprising administering into a distentable organ or tissue of the subject with a pharmaceutical composition comprising a formulation disclosed herein and using a dosing regimen designed to improve drug-target interaction and overall efficacy. The disclosed methods may pretreat a distentable organ with a formulation and set of conditions that increases and improves the binding and affinity of the drug to its target and/or improves efficacy by secondary mechanisms. The disclosed methods may treat a distentable organ with a drug formulation and set of conditions that increases and improves the binding and affinity of the drug to its target and/or improves efficacy by secondary mechanisms. The disclosed methods may post-treat a distentable organ with a formulation and set of conditions that increases and improves the binding and affinity of the drug to its target and/or improves efficacy by secondary mechanisms. The disclosed methods use pre-dosing, dosing and post-dosing formulations and conditions may include volume, temperature, pH, viscosity, dielectric, ionic strength, monovalent ions, divalent ions, excipients that act as volume excluders, detergents, chaotropic agents, stabilizers.

[0069] In one aspect, provided herein is a method to treat disease in a distentable organ in a subject. The method comprises administering into the organ of the subject a pharmaceutical composition comprising formulations as demonstrated to improve drug-target interaction and overall efficacy (Figures 2-5). The methods may use any of the set of conditions shown to increase and improve the binding and affinity of the drug to its target and/or improve efficacy. Figures 2-5 experimentally demonstrate the following conditions enable binding between A-dmDT390-EGF (of SEQ ID NO: 1) or A-dmDT390-EGF-Kd+ (of SEQ ID NO: 2) and EGFR:

HBS (10mM HEPES pH 7.4, 150mM NaCl, and 0.05% Surfactant P20)

PBS (10 mM Sodium Phosphate pH 7.4, 150 mM NaCl, 0.05% Surfactant P20)

150 – 500 mM NaCl

0-20 mM MgCl_2

10-500 mM NaPO_4

0-200 mM Glucose

RMPI (all constituents)

0-1mM EDTA

pH 6 – 8.

[0070] One of skill in the art would not have expected to see pH 7.4 was so significantly improved as compared to pH 6 (especially given that urine typically exhibits a pH below 6. Also surprising was the improved binding in the absence of divalents such as MgCl_2 , or with minimal amounts of divalents, for example, less than about 20 mM, or less than about 10 mM, or less than about 2 mM divalents. And finally, the improved binding with increasing glucose would not be expected by one of skill in the art.

[0071] First demonstrated herein, a pH at or above 7.4 provides improved binding (Figure 3), for example at least about pH 7.0, about 7.2, about 7.4, about 7.6, about 7.8, about 8.0, and so on, and from Figure 4, monovalent ions, such as NaCl show an optimum of the tested conditions found at about 10 mM, for example, about 5 mM to about 15 mM, or about 8 mM to about 12 mM, or about 5 mM, or about 6 mM, or about 7 mM, or about 8 mM, or about 9 mM, or about 10 mM, or about 11 mM, or about 12 mM, or about 13 mM, or about 14 mM, or about 15 mM, while the ionic strength of buffering agents, such as NaPO_4 are effective with an optimum of the tested conditions found at about 150 mM, for example, at about 145 mM, about 148 mM, about 152 mM, about 154 mM, at about 140 to about 160 mM, or about 145 mM to about 155 mM, or about 150 mM to about 160 mM, or about 140 mM to about 150 mM, and so on, and divalent ions, such as MgCl_2 are most effective if they are absent. The absence of divalent ions can be achieved by using chelation agents such as bisphosphonates or EDTA (as was present up to 35 μM in the drug solution of SPR experiments). The presence of about 10 mM to about 150 mM glucose, or about 20 mM to about 120 mM glucose, or at least about 10 mM glucose, at least about 50 mM glucose, or at least about 100 mM glucose improved binding, for example, in the tested conditions shown in Figure 5.

[0072] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled formulation comprising: 10 mM PO_4 , 150 mM NaCl , pH 8.0.

[0073] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled formulation comprising: 10 mM PO_4 , 150 mM NaCl , pH 8.0, 50-200 mM Glucose.

[0074] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled formulation comprising: 10 mM PO₄, 150 mM NaCl, pH 8.0, 0.5 - 20 mM EDTA.

[0075] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled formulation comprising: 10 mM PO₄, 150 mM NaCl, pH 8.0, 20 mM zoledronate (or any bisphosphonate).

[0076] In one aspect, provided herein is a method providing colorization agents used to monitor and assess the degree of dilution caused by addition of urine during the treatment time and to maintain conditions within optimal ranges.

[0077] In one aspect, provided herein is a method that uses known or published binding conditions as elements of strategies for improving efficacy in formulations and conditions development, or as elements of the formulations and/or conditions themselves.

[0078] In one aspect, provided herein is a method that uses cell-based assays and/or in vivo assessment of binding conditions as elements of strategies for improving efficacy in formulations and conditions development, or as elements of the formulations and/or conditions themselves.

[0079] In one aspect, provided herein is a method that uses cell-based assays and/or in vivo assessment of pre-dosing, dosing, and post-dosing conditions as elements of strategies for improving efficacy in formulations and conditions development, or as elements of the formulations and/or conditions themselves.

[0080] In one embodiment the method comprises treating the subject with bladder cancer with an instilled volume (75mL – 1500mL) that maximizes exposure of cellular surface targets to a fusion protein-toxin, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target.

[0081] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled volume (75mL – 1500mL) that minimizes dilution of the drug formulation designed to improve binding to cellular surface targets by a fusion protein-toxin drug.

[0082] In one embodiment, the method comprises treating the subject with bladder cancer with an instilled formulation and conditions that maximizes binding to cellular surface targets by a fusion protein-toxin drug, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target.

[0083] In one embodiment, the method comprises treating the subject with bladder cancer with a pre-dose instilled formulation under conditions that maximizes binding to cellular surface

targets by a fusion protein-toxin drug, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target, by disruption or removal of the glycocalyx or other inhibitors of binding.

[0084] In one embodiment, the method comprises treating the subject with bladder cancer with a pre-dose instilled formulation under conditions that maximizes binding to cellular surface targets by a fusion protein-toxin drug, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target, and/or facilitates secondary mechanisms (other than binding enhancement) that improve efficacy.

[0085] In one embodiment, the method comprises treating the subject with bladder cancer with a post-dose instilled formulation under conditions that maximizes binding to cellular surface targets by a fusion protein-toxin drug, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target, and/or facilitates secondary mechanisms (not binding enhancement) that improve efficacy.

[0086] In one embodiment, the method comprises treating the subject with bladder cancer with a pre-dosing and/or dosing and/or post-dose instilled formulation with a instillate hold-time to maximize binding to cellular surface targets by a fusion protein-toxin drug, such as Diphtheria-EGF to its EGFR target, or epCAM-ToxinA to its EpCAM target, and/or facilitates secondary mechanisms (not binding enhancement) that improve efficacy.

Examples

[0087] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, room temperature is about 25°C, and pressure is at or near atmospheric.

Example 1

[0088] Binding measurements and analyses of two DTEGF fusion-toxins to EGF by Surface Plasmon Resonance (SPR) were determined. The data was collected using a 300 (s) association phase and a 900 (s) dissociation phase. Kinetic rate coefficients were recovered from a binding analysis experiment performed with a Biacore 3000 biosensor. Six concentrations of analyte ranging between 300 nM to 1.23 nM were run in duplicate against captured rhu EGFR (Fc). The association and dissociation phase data were globally fit to a 1:1 binding model to determine the association rate coefficient (k_a), dissociation rate coefficient (k_d) and the R_{max} value. Results are reported as the global fits to 1:1 binding model $\pm$ standard deviation. See Figure 2.

Example 2

[0089] SPR assay to screen buffer conditions - NaCl, pH, buffer strength, and divalent cation for the A-dmDT390-EGF and A-dmDT390-EGF-Kd+ / rhuEGFR interactions. The rhuEGFR capture at ~ 275 RU. A single of 300nM concentration of each analyte was injected. ¹HBS P 10 mM Hepes, 150 mM NaCl, 0.05% Surfactant P20, pH 7.4; ²PBS P 10 mM Sodium Phosphate, 150 mM NaCl, 0.05% Surfactant P20, pH 7.4. See Figure 3.

Example 3

[0090] A-dmDT390-EGF (BO5) and A-dmDT390-EGF-Kd+ (BO1) treatments for 15 minutes demonstrate potent cell killing on bladder cancer cell line HTB9. See Figure 4.

Example 4

[0091] HTB9 cells seeded in 96 wells plate on day1 in RPMI1640 with 10% FCS, attached for 2 days then treated with 5ng/mL A-dmDT390-EGF with the indicated glucose concentration for 2 hours. Removed the treatment, washed twice with 50 ul medium then added 100ul medium and incubate for 72 hours, viability was accessed with MTT assay. Each condition was done with triplicate. See Figure 5.

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[0092] All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (*e.g.*, Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicant to relate to each and every individual publication, database entry (*e.g.*, Genbank sequences or GeneID entries), patent application, or patent identified even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

We claim:

1. A composition comprising:
 - a. A diphtheria toxin-epidermal growth factor (DT-EGF) fusion protein of structure A-X-Y-Z, wherein:
 - b. A is 0-5 amino acid residue N-terminal addition to the front of the diphtheria toxin sequence;
 - c. X is a diphtheria toxin fragment or mutated fragment that maintains catalytic activity;
 - d. Y is sequence of amino acids from 0 – 20 aa in length that connects the carboxy terminus of X to the amino terminus of Z; and
 - e. Z is epidermal growth factor or mutant thereof that maintains binding affinity for epidermal growth factor receptor.
2. The composition of claim 1, wherein the composition is in a formulation having a pH of at least about 7.0, at least about 7.4, or at least about 8.
3. The composition of claim 1, wherein the composition is in a formulation that comprises at least about 10 mM glucose, at least about 50 mM glucose, or at least about 100 mM glucose.
4. The composition of claim 1, wherein the composition is in a formulation having a minimal amount of MgCl₂, or less than about 20 mM MgCl₂, or less than about 10 mM MgCl₂, or less than about 2 mM MgCl₂.
5. The composition of claim 1, wherein the composition is formulated with 10 mM PO₄ and 150 mM NaCl at pH 8.0.
6. The composition of claim 1, where A is a single alanine.
7. The composition of claim 1, where X is (glycosylation sites removed)

gaddvvdss ksfvmenfas yhgtpgyvd siqkgiqkpk sgtqgnyddd
 wkgfystdnk ydaagysvsn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls
 lteplmeqvg teefikrfgd gasrvvslp faegssvey innweqakal sveleinfet
 rgkrqdamy eymaqacagn rvrrsvgssl scinldwdvi rdtktkies lkehgpi knk
 msespaktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya awavnvaqvi
 dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaipvlge
 lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf (SEQ ID NO: 1).

8. The composition of claim 1 where X is

gaddvvdss ksfvmenfss yhgtpgyvd siqkgiqkpk sgtqgnyddd
 wkgfystdnk ydaagysvsn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls
 lteplmeqvg teefikrfgd gasrvvslp faegssvey innweqakal sveleinfet
 rgkrqdamy eymaqacagn rvrrsvgssl scinldwdvi rdtktkies lkehgpi knk
 msespnktvs eekakqylee fhqtalehpe lselktvtgt npvfaganya awavnvaqvi
 dsetadnlek ttaalsilpg igsvmgiadg avhhnteeiv aqsialsslm vaqaipvlge
 lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf (SEQ ID NO: 2)

9. The composition of claim 1 where X is a modified sequence that maintains catalytic activity while also evading immune response.

10. The composition of claim 1 where Y is

- a. ha,
- b. pw,
- c. aa,
- d. lp, or
- e. gg.

11. The composition of claim 1 where Y is

- a. A short sequence of 0 – 20 amino acids designed to be targeted by lysosomal or intracellular proteases

- b. A short sequence of 0 – 20 amino acids designed to minimize negative or inhibitory interactions of X and Z sequences.
12. The composition of claim 1 where Z is wild type human EGF sequence.
13. The composition of claim 1 where Z is:
 - a. wnsYsecp PsYdgyclhd gvcRyieald Syacncvgy Agercqyrdl RwwGRr (SEQ ID NO: 3); or
 - b. any mutant that increases affinity for the EGF receptor.
14. The composition of claim 1 comprising:

agaddvvdss ksfvmenfas yhgtpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk
ydaagysvdn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd
gasrvvlslp faegssvey innweqakal sveleinfet rgkrqdamy eymaqacagn
rvrrsvgssl scinldwdvi rdktktkies lkehgpi knk msepaktvs eekakqylee fhqtalehpe
lselktvtgt npvfaganya awavnvaqvi dsetadnlek ttaalsilpg igsvmgia dg avhhnteeiv
aqisialsslm vaqaipvlge lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsYsecp
PsYdgyclhd gvcRyieald Syacncvgy Agercqyrdl RwwGRr (SEQ ID NO: 6).
15. The composition of claim 1 comprising:

agaddvvdss ksfvmenfas yhgtpgyvd siqkgiqkpk sgtqgnyddd wkgfystdnk
ydaagysvdn enplsgkagg vvkvtypglt kvlalkvdna etikkelgls lteplmeqvg teefikrfgd
gasrvvlslp faegssvey innweqakal sveleinfet rgkrqdamy eymaqacagn
rvrrsvgsslscinldwdvi rdktktkies lkehgpi knk msepaktvs eekakqylee fhqtalehpe
lselktvtgt npvfaganya awavnvaqvi dsetadnlek ttaalsilpg igsvmgia dg avhhnteeiv
aqisialsslm vaqaipvlge lvdigfaayn fvesiinlfq vvhnsynrpa yspghktqpf lpwnsdsecp
lshdgyclhd gvcmyieald kyacncvgy igercqyrdl kwwelr (A-dmDT390-EGF)
(SEQ ID NO: 5).
16. A method to treat cancer in a distentable organ with an instilled volume that maximizes exposure of cellular surface targets to a targeted drug treatment.

17. A method to treat cancer in a distentable organ with an instilled volume comprised of a formulation and set of conditions that maximizes exposure of cellular surface targets to a targeted drug treatment.
18. A method to treat cancer in a distentable organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that maximizes exposure of cellular surface targets to a targeted drug treatment.
19. A method to treat cancer in a distentable organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that removes constituents that inhibit binding and exposure of cellular surface targets to a targeted drug treatment.
20. A method to treat cancer in a distentable organ with an instilled volume comprised of a formulation and set of conditions in a pre-dosing treatment that maximizes treatment efficacy of the targeted drug treatment by mechanisms other than improved binding of drug to target.
21. A method to treat cancer in a distentable organ with an instilled volume comprised of a formulation and set of conditions in a post-dosing treatment that maximizes treatment efficacy of the targeted drug treatment by mechanisms other than improved binding of drug to target.
22. The method of any one of claims 16-21 where the cancer is in the bladder.
23. The method of any one of claims 16-21 where the cancer is in the pleura.
24. The method of any one of claims 16-21 where the cancer is in the uterus.
25. The method of any one of claims 16-21 where the cancer is in the peritoneum.

26. The method of any one of claims 16-21 where the cancer is in the omentum.
27. The method of any one of claims 16-21 where the cancer is in the eye.
28. The method of any one of claims 16-21 where the targeted drug treatment is a protein-toxin fusion of Diphtheria toxin to Epidermal Growth factor.
29. The method of any one of claims 16-21 where the targeted drug treatment is a protein-toxin fusion of Psuedomonous ExoToxin A to EpCAM.
30. The method of claim any one of claims 16-21 where the targeted drug treatment is a protein-toxin fusion is ONTAK.
31. A pharmaceutical composition of any one of claims 1-15 for use in a method of any one of claims 16-30.
32. A pharmaceutical composition of any one of claims 1-15 for use in the manufacture of a medicament for use in a method of any one of claims 16-30.
33. The pharmaceutical composition of claim 32, comprising SEQ ID NO: 5 or SEQ ID NO: 6.

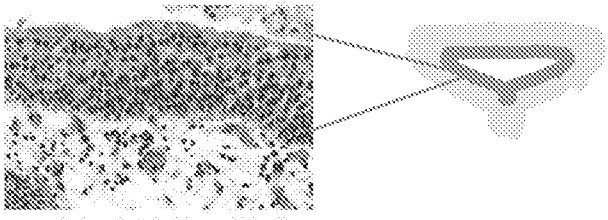
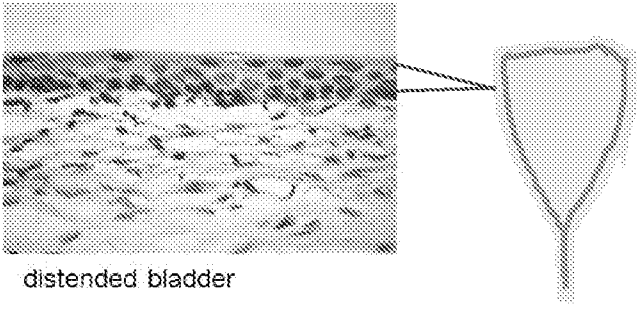
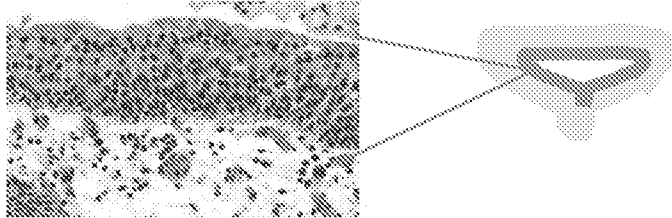
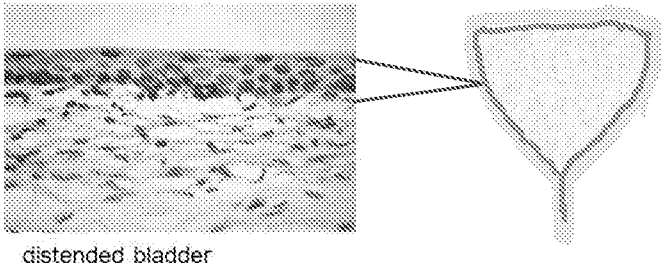
Pre-dosing Wash	 non-distended (relaxed) bladder	Empty bladder
	 distended bladder	Instill 500 mL and hold for 15 minutes Sterile isotonic saline w/ 10 mM Citrate pH6.0, 37°C, 0.01% Sodium dodecylsulfate Empty bladder Flush and empty 3x with 500 mL Sterile Isotonic saline (Breakdown and removal of glycolyx other binding inhibitors)
Dosing	 non-distended (relaxed) bladder	Empty bladder: (Urothelium cleaned of inhibitors to drug binding)
	 distended bladder	Instill 500 mL – maintain continuous irrigation for 24 hours maintaining ~500 mL with the following: 10 uM DT389EGF 10 mM Sodium Phosphate pH 7.2 200 mM NaCl, 1 mM MgCl ₂ pH 7.2, 37°C, (Conditions set to maximize interaction of EGF and EGFR)

Figure 1A

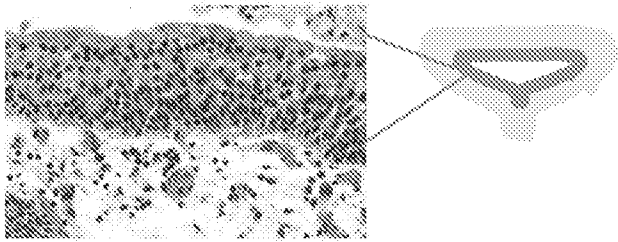
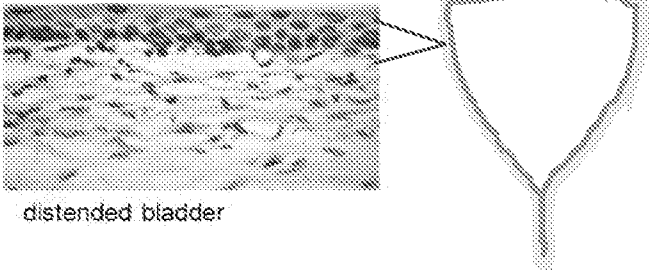
Post-dosing Conditioning	 non-distended (relaxed) bladder	Empty bladder: (Drug tightly bound to EGFR receptor)
	 distended bladder	Instill 500 mL and hold for 1 hour: 0.25% Acetic Acid pH 4.5 40 °C, Empty bladder Flush and empty 3x with 500 mL Sterile Isotonic saline (Drug release from endosome facilitated by acid and the heat induced increase in chaperone proteins – increased cytoplasmic drug concentration and activity)

Figure 1B

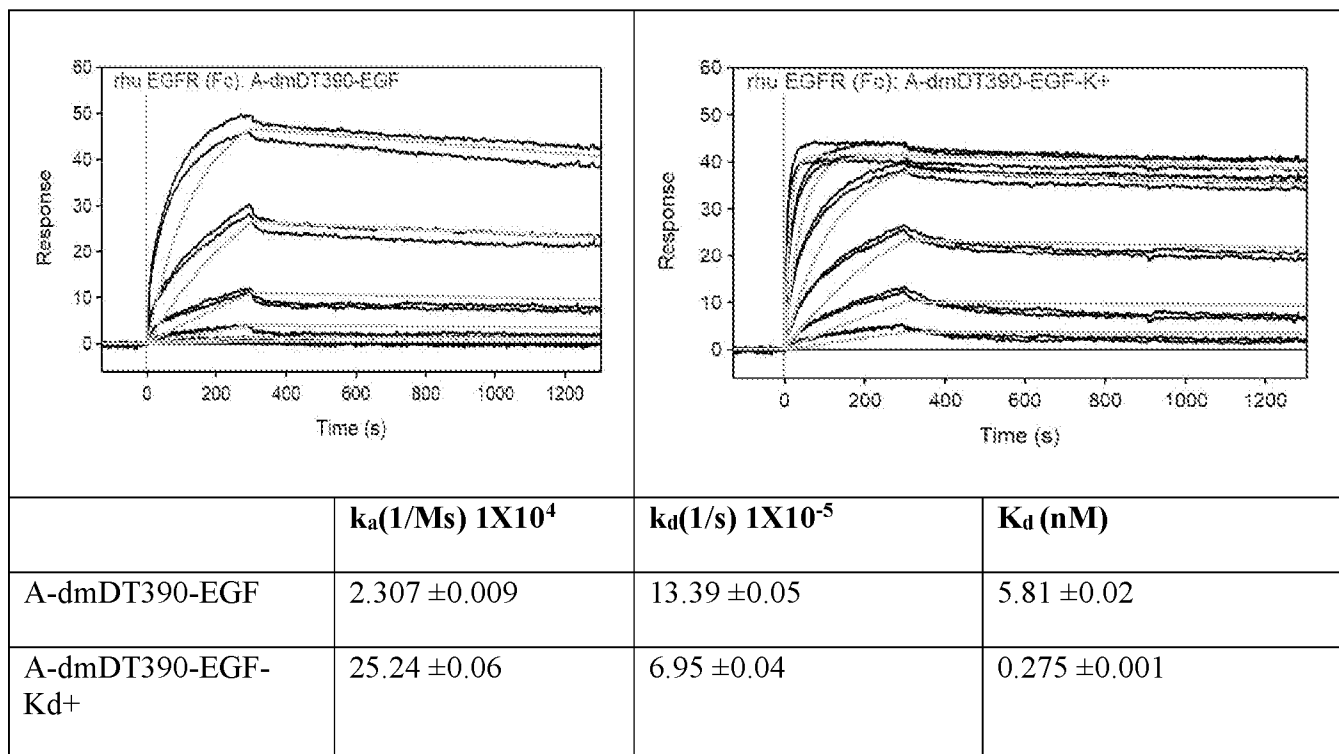


Figure 2

Buffer Condition	A-dmDT390-EGF				A-dmDT390-EGF (Kd+)			
	$k_a(1/Ms)$	$k_d(1/s)$	Rmax (RU)	KD (nM)	$k_a(1/Ms)$	$k_d(1/s)$	Rmax (RU)	KD (nM)
¹ HBS P 150 mM NaCl	5.75E+04	1.39E-04	82.48	2.42	4.33E+05	4.52E-05	73.64	0.104
HBS P 250 mM NaCl	5.45E+04	1.55E-04	83.22	2.83	4.82E+05	5.10E-05	74.20	0.106
HBS P 500 mM NaCl	7.74E+04	1.43E-04	88.58	1.84	5.82E+05	6.82E-05	74.25	0.117
² PBS P pH 6.0	1.78E+04	1.81E-03	52.80	102	2.73E+05	3.51E-04	55.04	1.280
PBS P pH 7.4	6.36E+04	1.17E-04	79.53	1.83	4.79E+05	3.66E-05	53.61	0.076
PBS P pH 8.0	7.45E+04	1.07E-04	76.37	1.43	5.81E+05	4.07E-05	54.03	0.070
PBS P 10 mM NaPO4	6.36E+04	1.17E-04	79.53	1.83	4.79E+05	3.66E-05	53.61	0.076
PBS P 100 mM NaPO4	6.07E+04	8.42E-05	78.62	1.39	5.90E+05	1.97E-05	55.48	0.033
PBS P 500 mM NaPO4	3.18E+04	8.45E-05	70.56	2.66	3.07E+05	7.85E-05	70.61	0.256
PBS P	6.36E+04	1.17E-04	79.53	1.83	4.79E+05	3.66E-05	53.61	0.076
PBS P 2 mM MgCl2	5.83E+04	1.28E-04	71.84	2.19	3.84E+05	3.80E-05	49.08	0.099
PBS P 20 mM MgCl2	7.10E+04	1.63E-04	83.53	2.30	3.15E+05	4.81E-05	53.48	0.153

Figure 3

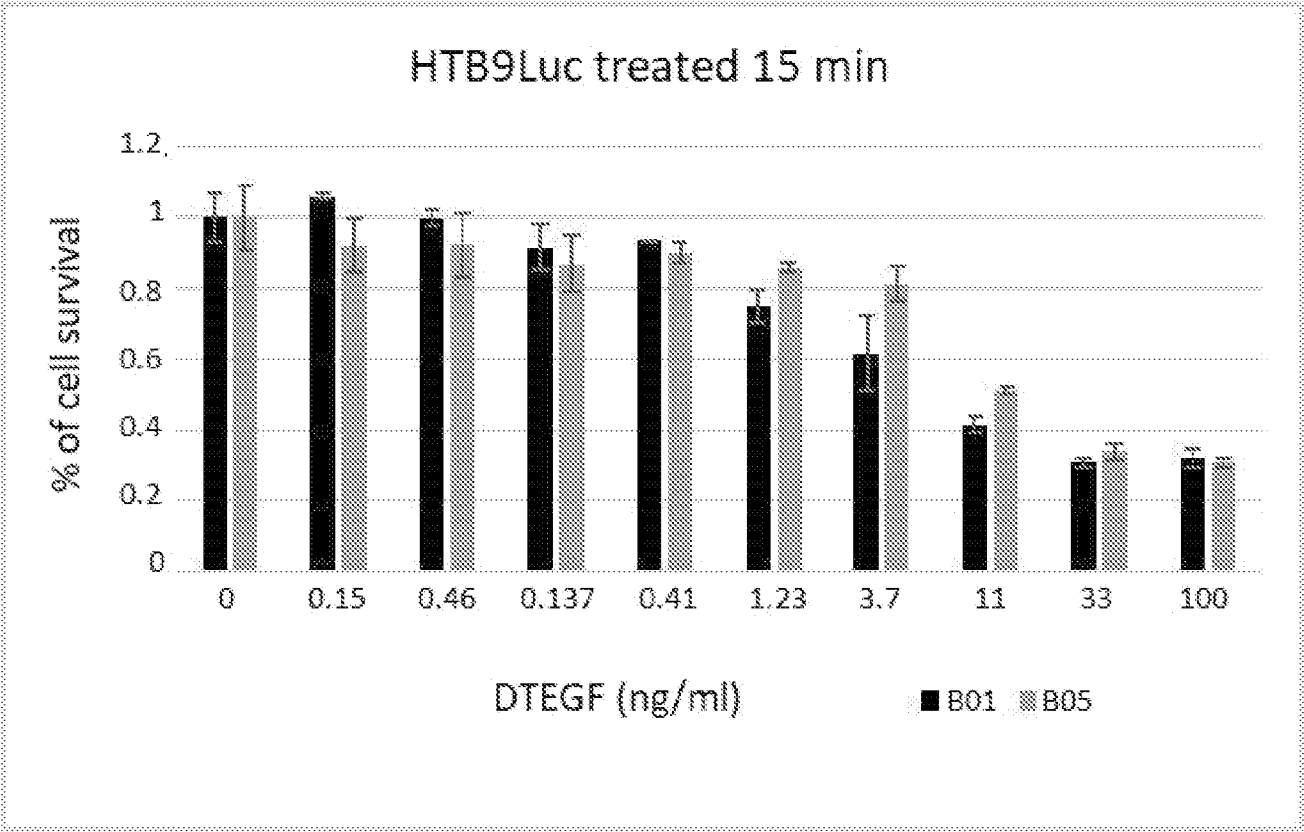


Figure 4

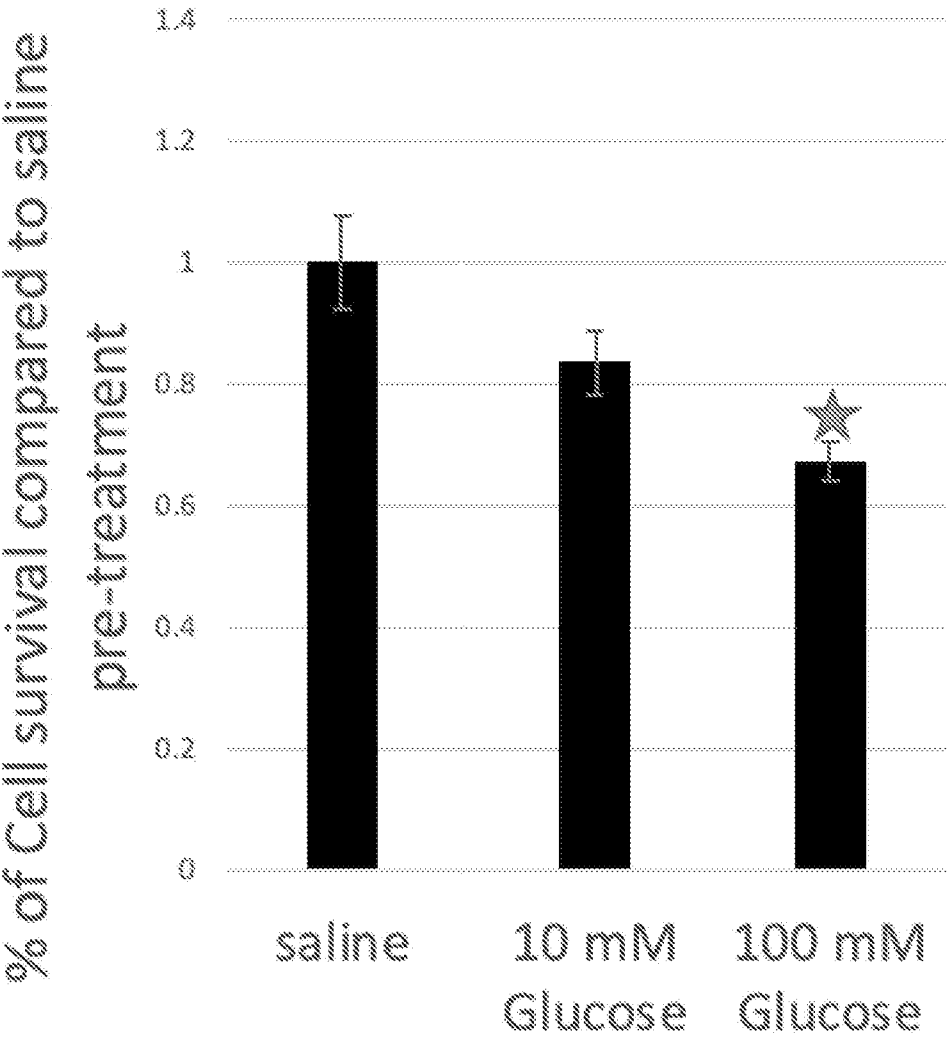


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/029396

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61P35/00 C07K14/34 C07K14/485 A61K38/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61P C07K C12R A61K

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

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

EPO-Internal, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HORITA HENRICK ET AL: "EGFR-targeted diphtheria toxin stimulates TRAIL killing of glioblastoma cells by depleting anti-apoptotic proteins", JOURNAL OF NEURO-ONCOLOGY, KLUWER, BOSTON, US, vol. 95, no. 2, 1 November 2009 (2009-11-01), pages 175-184, XP036258957, ISSN: 0167-594X, DOI: 10.1007/S11060-009-9914-4 [retrieved on 2009-11-01] cited in the application abstract right column; page 175 page 177 - page 178 ----- -/--	1,11,12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

15 July 2020

Date of mailing of the international search report

16/09/2020

Name and mailing address of the ISA/

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Steinheimer, K

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2020/029396

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/047135 A2 (UNIV COLORADO REGENTS [US]; FLAIG THOMAS [US] ET AL.) 21 April 2011 (2011-04-21)	1,6,7, 11,12, 31-33
Y	paragraph [0033] - paragraph [0036] claims 1-6	1-15
X	WO 2017/059270 A1 (UNIV MINNESOTA [US]; VALLERA DANIEL ATTILIO [US]) 6 April 2017 (2017-04-06)	1,11,12
Y	page 18 claims 4, 10 page 6 - page 7 page 17	1-15
X	JP 2004 155776 A (OSAKA IND PROMOTION ORG) 3 June 2004 (2004-06-03)	1,11,12
Y	the whole document	1-15, 31-33
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Y	paragraph [0064] sequences 3-4	1-15, 31-33
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Y	example 1 figures 1A, 2A, 3	1-15
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X	WO 2007/109673 A2 (UNIV STANFORD [US]; MASSACHUSETTS INST TECHNOLOGY [US] ET AL.) 27 September 2007 (2007-09-27)	1,11-13
	sequence 36 page 24 - page 27	
X	WO 2005/052129 A2 (ANJIN CORP [US]; HARRISON ROBERT J [US]; VANDERSPEK JOANNA C [US]) 9 June 2005 (2005-06-09)	1,11,12
Y	table 2 sequence 2	7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/029396

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

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

1-15, 31-33

Remark on Protest

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15, 31-33

Composition comprising the product as defined in claim 1 and its use in a pharmaceutical composition for therapeutic use as defined in claims 16-30

2. claims: 16-30

Method to treat cancer in a distentable organ as defined in claims 16-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2020/029396

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