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(54) **Title:** STABLE COLLOIDAL DRUG AGGREGATES AND METHODS OF MANUFACTURE AND USE THEREOF

(57) **Abstract:** The present application provides a colloid drug aggregate composition and methods of use and manufacture thereof. While the formation of colloidal aggregates leads to artifacts in early drug discovery, their composition makes them attractive as nanoparticle formulations for targeted drug delivery. The present application provides an acid-responsive composition comprising: a colloidal aggregate of one or more drugs and a stabilizing agent, wherein the colloidal aggregate disrupts, dissolves or disassembles when the acid-responsive composition is in an acid environment having a pH of less than 7.4. The colloidal aggregate of the composition will disassemble upon contact with acid or upon introduction to an acidic environment, such as is found in the endosomes of cells. This approach makes this composition an attractive vehicle for drug delivery to a target site in a subject or to cells.



STABLE COLLOIDAL DRUG AGGREGATES AND METHODS OF MANUFACTURE AND USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] The present application claims priority from United Kingdom Patent Application No. GB1908716.2, filed June 18, 2019, which is incorporated herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[002] This invention was made with government support under grant no. GM071630 awarded by The National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[003] The present application pertains to the field of pharmaceuticals. More particularly, the present application relates to colloidal drug aggregates, and methods of manufacture and uses thereof.

INTRODUCTION

[004] Some small-molecule drugs spontaneously self-assemble under aqueous conditions to form colloidal aggregates.¹⁻³ The formation of these colloids is governed by a critical aggregation concentration (CAC) and generally occurs when water is added to a solution of drug in a water-miscible organic solvent.⁴ Although colloidal drug aggregates are best known for causing false hits in early drug discovery,⁵⁻⁷ recent efforts have aimed to stabilize these drug-rich particles for delivery.⁸⁻¹⁰

[005] Development of stable colloidal drug aggregates has paradoxically created a new problem: colloid-associated drug does not permeate the cell membrane to interact with intracellular targets.^{11,12} One method to overcome this issue is to disrupt the particles to yield drug monomers.^{12,13} Colloid dissolution has traditionally been accomplished by adding

detergent; however, this strategy is more useful *in vitro* than *in vivo*, where toxic excipients are dose-limited.

[006] A need remains for colloidal drug formulations that do not require chemical modification for aggregation and that minimize or avoid the use of toxic excipients for stabilization.

[007] The above information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[008] An object of the present application is to provide stable colloidal drug aggregates and methods of manufacture and use thereof. In accordance with an aspect of the present application, there is provided an acid-responsive composition comprising: a colloidal aggregate of one or more drugs and a stabilizing agent, wherein the colloidal aggregate disrupts, dissolves or disassembles when the acid-responsive composition is in an acid environment having a pH of less than 7.4 (such as, a pH of less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4). In accordance with one embodiment, the acid-responsive composition comprises: a colloidal aggregate of one or more drugs and a stabilizing agent, wherein (a) at least one of the one or more drugs is an ionizable drug or ionizable drug analogue, wherein the conjugate acid of the ionizable drug or drug analogue has a pKa of at least 4, preferably at least 4.5 or at least 5, or at least 5.5, or at least 6, or at least 6.5; (b) the stabilizing agent is an acid-responsive stabilizing agent such that the stabilizing agent undergoes a morphological and/or functional change when pH is reduced to less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4; or (c) both (a) and (b).

[009] In accordance with an embodiment of the present application, there is provided an acid-responsive composition comprising: a colloidal aggregate of one or more ionizable

drugs or ionizable drug analogues; and a stabilizing agent, wherein the conjugate acid of the ionizable drug or drug analogue has a pKa of at least 4, preferably at least 4.5 or at least 5, or at least 5.5, or at least 6, or at least 6.5. In another embodiment of the present application, there is provided an acid-responsive composition comprising a colloidal aggregator, which is either an ionizable or non-ionizable drug or drug analogue; and an acid-responsive stabilizing agent that undergoes a morphological and/or functional change when the pH is reduced to less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4.

[0010] In certain embodiments the stabilizing agent is a protein (e.g. an antibody or antibody fragment), a polymer, a colloid-forming compound (e.g., vitamin E) or another colloid-forming drug (e.g., fulvestrant). In the example in which the stabilizing agent is another colloid-forming drug, the other colloid-forming drug is, optionally, not ionizable at a pH of 4 or less. Optionally, the stabilizing protein is IgG, trastuzumab, albumin, transferrin, or an attenuated bacterial toxin (such as attenuated diphtheria toxin or derivatives thereof). Suitable stabilizing polymers include polymeric surfactants, such as, but not limited to, UP80, PLAC-PEG, Brij 58, F127, Vitamin E-PEG, F68, or Brij L23.

[0011] In accordance with certain embodiments, the stabilizing agent compromises the integrity of plasma membranes in acidic environments (e.g., the stomach or a lysosome or an endosome of a cell), thereby allowing colloidal aggregates or their contents to be transported out of the acidic environment.

[0012] In accordance with certain embodiments, the colloidal aggregate is disrupted upon contact of the composition with an acid or introduction of the composition to an acidic environment (e.g., the stomach or a lysosome or an endosome of a cell), and the one or more drug or drug analogue is released as a result of the disruption of the colloid.

[0013] In accordance with certain embodiments, the colloidal aggregate comprises a targeting compound for delivery of the composition to a target site. The targeting compound can be, for example, a protein (e.g., transferrin), an antibody (e.g. trastuzumab), an attenuated bacterial toxin (such as attenuated diphtheria toxin or derivatives thereof), or another molecule that selectively binds to a cell receptor. Optionally, the targeting compound also

functions as the stabilizing agent, or acts together with another stabilizing agent to aid in stabilization of the colloidal aggregate.

[0014] In a specific embodiment, the ionizable drug is lapatinib and the composition further comprises a stabilizing compound, which is fulvestrant, and a targeting compound, which is transferrin.

[0015] In another specific embodiment, the ionizable drug is lapatinib and the composition further comprises a stabilizing compound, which is a combination of fulvestrant and a polymeric surfactant (e.g., PLAC-PEG).

[0016] In accordance with certain embodiments, the colloidal drug aggregate composition comprises an ionizable drug analogue that comprises a drug molecule (e.g., sorafenib or fulvestrant) chemically modified to include an ionizable moiety. Optionally, the ionizable drug analogue is itself pharmaceutically active. Alternatively, the analogue functions as a prodrug such that following ionization and release from the colloid, the analogue is modified (e.g., by cleavage of the ionized moiety) *in vivo* to form the active drug molecule.

[0017] Another aspect of the present application provides a method of drug therapy comprising administration of the colloidal drug aggregate composition to a subject in need thereof.

BRIEF DESCRIPTION OF FIGURES AND TABLES

[0018] For a better understanding of the application as described herein, as well as other aspects and further features thereof, reference is made to the following description which is to be used in conjunction with the accompanying drawings and tables.

[0019] Figure 1: Curve-fitting algorithm identifies the critical aggregation concentration of lapatinib at **A)** pH 7.4 and **B)** pH 5.5. Lapatinib fluorescence is proportional to the amount of colloidal lapatinib, which increases linearly with concentration once the CAC is reached.

[0020] Figure 2: Lapatinib is not stabilized by polymeric excipients. Colloidal lapatinib at 50 μ M was formulated with 0.01 mg/mL stabilizer in water and incubated at 37°C. DLS

characterization of **A**) size and **B**) scattering intensity shows precipitation over 24 h, even in formulations that were initially stable.

[0021] Figure 3: Lapatinib is not stabilized in PBS by co-aggregation with Congo red (CR). Colloidal lapatinib at 50 μM was formulated with varying amounts of Congo red in PBS and incubated at 37°C. DLS characterization of **A**) size and **B**) scattering intensity shows precipitation over 24 h, even in formulations that were initially stable ($n=3$, mean $\pm$ SD).

[0022] Figure 4: Transmission electron micrographs of colloidal drug aggregates. Colloids were formulated with 25 μM each of fulvestrant and lapatinib with 0.01 mg/mL UP80 in PBS. Three representative darkfield images show the spherical morphology and 100–200 nm size of these colloidal drug aggregates.

[0023] Figure 5: Zeta potential of stabilized lapatinib and fulvestrant colloids (fulvestrant alone shown in the lower bars, with negative zeta potential). Bare lapatinib-containing colloids have a highly positive surface charge, which is mitigated by stabilizers ($n=3$, mean $\pm$ SD).

[0024] Figure 6: Colloidal lapatinib is stabilized by co-aggregation with fulvestrant and polymeric surfactant. Colloidal drug aggregates were formulated with 50 μM total drug, and 0.01 mg mL⁻¹ poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (PLAC-PEG) in phosphate buffered saline. A 1:3 mol ratio of lapatinib:fulvestrant was selected for its stability against **A**) growth and **B**) precipitation as measured by dynamic light scattering. **C**) Fluorescence measurement ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 450 \text{ nm}$) confirmed the presence of lapatinib in these co-colloids ($n = 3$, mean $\pm$ SD).

[0025] Figure 7: Colloidal stability characterized by dynamic light scattering, comparing PLAC-PEG-stabilized co-colloids containing different ratios of fulvestrant and lapatinib. Colloids were formulated in PBS with 50 μM total drug and 0.01 mg/mL PLAC-PEG. The stable lapatinib–fulvestrant colloids were confirmed to be relatively monodisperse ($\text{PDI} < 0.2$) by DLS ($n=3$, mean $\pm$ SD).

[0026] Figure 8: Stability characterization by dynamic light scattering of lapatinib and fulvestrant co-colloids stabilized with different amounts of transferrin. Concentrations of 12.5

μM lapatinib and $37.5 \mu\text{M}$ fulvestrant were used in this study, and transferrin was introduced in the water addition step. DLS analysis of **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** size dispersity shows that transferrin stabilizes these colloids in a concentration-dependent manner ($n=3$, mean $\pm$ SD).

[0027] Figure 9: Stability characterization by dynamic light scattering (DLS) of lapatinib and fulvestrant co-colloids stabilized with transferrin. Co-colloids comprised concentrations of $50 \mu\text{M}$ ($29 \mu\text{g/mL}$) lapatinib and $150 \mu\text{M}$ ($91 \mu\text{g/mL}$) fulvestrant, and $100 \mu\text{g/mL}$ transferrin. DLS analyses show that transferrin stabilizes these high-concentration colloids: **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) ($n=3$, mean $\pm$ SD).

[0028] Figure 10: Stability characterization by dynamic light scattering of colloids formulated in cell culture media. Colloids were formulated as specified in Table 4. DLS analysis of **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) demonstrate stability in this environment ($n=3$, mean $\pm$ SD)

[0029] Figure 11: Colloids persist after acidification. Colloids were formulated with $37.5 \mu\text{M}$ fulvestrant, $12.5 \mu\text{M}$ lapatinib, and either 0.05 mg/mL transferrin or 0.01 mg/mL PLAC-PEG, as indicated in the legend. Dynamic light scattering analyses demonstrate persistence of the colloids after acidification: **A)** hydrodynamic diameter, **B)** scattering intensity, and **C)** polydispersity index (PDI) ($n=3$, mean $\pm$ SD).

[0030] Figure 12: Lapatinib release from stable lapatinib-fulvestrant co-colloids is triggered by acidic conditions. Colloids were formulated with $12.5 \mu\text{M}$ lapatinib, $37.5 \mu\text{M}$ fulvestrant, and 0.01 mg mL^{-1} poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (PLAC-PEG) in PBS. **A)** Lapatinib was released from the co-colloids with decreasing pH whereas **B)** fulvestrant was retained within the colloids. **C)** Lapatinib release from co-colloids was triggered by acidification to pH 5.5 (colored arrows) whereas **D)** fulvestrant release was unaffected ($n \geq 3$, mean $\pm$ SD, one-way ANOVA with Tukey's post-hoc test, ns $p > 0.05$, **** $p < 0.0001$ compared to initial release at pH 7.4).

[0031] Figure 13: Lapatinib release from stable lapatinib-fulvestrant co-colloids is triggered by acidic conditions. Colloids were formulated with $50 \mu\text{M}$ lapatinib, $150 \mu\text{M}$ fulvestrant,

and 0.04 mg/mL PLAC-PEG in PBS. **A)** Lapatinib was released from the co-colloids with decreasing pH whereas **B)** fulvestrant was retained within the colloids.

[0032] Figure 14: Lapatinib release from co-colloids is triggered by acidic conditions. Colloidal drug aggregates were formulated with 12.5 μ M lapatinib, 37.5 μ M fulvestrant, and 0.01 mg/mL PLAC-PEG in PBS supplemented with 10% FBS. The pH of these formulations were adjusted, followed by centrifugation to pellet out the colloids. Finally, the concentration of drug remaining in the supernatant was quantified. **A)** Lapatinib dissolved from the co-colloids under acidic conditions, whereas **B)** fulvestrant did not ($n \geq 3$, one-way ANOVA with Tukey's post-hoc test, ns $p > 0.05$, $*p < 0.05$, $***p < 0.001$ compared to pH 7.4, mean $\pm$ SD). These colloids were also incubated over time and acidified to pH 5.5 as indicated by the arrows; under these conditions **C)** lapatinib release was rapid and persisted, whereas **D)** the concentration of free fulvestrant was unaffected ($n = 3$, one-way ANOVAs with Tukey's post-hoc test, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$ compared to initial time point, mean $\pm$ SD).

[0033] Figure 15: Decoration of colloidal drug aggregates with transferrin enhances their endocytosis. MDA-MB-231-H2N cells were treated with colloids (Table 4) with the addition of 500 nM hydrophobic BODIPY dye. Images: cells were treated with lapatinib and fulvestrant colloids stabilized with either **A)** transferrin or **B)** poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (PLAC-PEG) for 1 h followed by fresh media for 23 h. The cells were labeled with CellMask™ Green (1x, 5 min) and imaged under live cell conditions. Transferrin-stabilized colloids were visualized as punctate structures inside the cells whereas PLAC-PEG-stabilized colloids were not observed inside the cells. **C)** Cells were treated for 3 h, and fluorescence of the BODIPY dye inside the cells was then analyzed by flow cytometry ($n = 3$ biological replicates, mean $\pm$ SD, two-way ANOVA with Tukey's post-hoc test, $***p < 0.001$, $****p < 0.0001$ compared to all other groups).

[0034] Figure 16: Colloids are visualized in punctate structures in cells only when stabilized by transferrin. Treatments were formulated as specified in Table 4 with the addition of 500 nM BODIPY dye (all except "Blank"). MDA-MB-231-H2N cells were treated in serum-free media for 3 h, then washed, fixed, and imaged. These representative confocal images show

450 nm lapatinib fluorescence (blue), 575 nm colloid dye fluorescence (green), and 488 nm transmission showing cell outlines.

[0035] Figure 17: Lapatinib-fulvestrant colloids are cytotoxic after endocytosis and subsequent endosome disruption. Colloidal drug aggregates were formulated as described in Table 4, with the addition of 2 μ M 7-aminoactinomycin D (7-AAD) to monitor endosome disruption in those experiments. Colloids composed of 50 μ M lapatinib and 150 μ M fulvestrant and stabilized with transferrin resulted in greater **A)** cytotoxicity and **B)** endosomal disruption (measured by amount of nuclear dye) than either co-colloids stabilized with PLAC-PEG or colloids of fulvestrant alone (150 μ M fulvestrant plus stabilizer) ($n = 9$ biological replicates and separate colloid formulations for the toxicity experiment and $n=3$ for the endosome disruption experiment, mean $\pm$ SD, two-way ANOVA with Tukey's post-hoc test, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$,). **C)** The top panel of images shows staining for Lapatinib and the bottom panel of images shows staining for 7-AAD. Nuclear 7-AAD fluorescence is visible after treatment with lapatinib-fulvestrant colloids stabilized with transferrin, demonstrating its endosomal escape. Permeabilized cells accumulate 7-AAD in their nuclei as expected whereas healthy cells treated with 7-AAD alone do not. The presence of cells in each region of interest was verified using the transmission channel prior to capturing these images.

[0036] Figure 18: Control images visualize 7-AAD nuclear fluorescence (red) in colloid-treated MDA-MB-231-H2N cells. Cells were treated with conditions outlined in Table 4 supplemented with 2 μ M 7-AAD. The presence of cells in each region of interest was verified using the transmission channel prior to capturing these images.

[0037] Figure 19: MDA-MB-231-H2N cells are still viable immediately after 3 h of treatment with colloids. Cells were treated identically to those used in viability and flow cytometry experiments depicted in Figure 17, then immediately assessed for viability ($n=3$, one-way ANOVA with Tukey's post-hoc test, $ns p > 0.05$, mean $\pm$ SD).

[0038] Figure 20: Lapatinib diffusion across an artificial lipid membrane is decreased under acidic conditions. Colloidal drug aggregates were formulated with 50 μ M lapatinib, 150 μ M fulvestrant, and 0.04 mg/mL PLAC-PEG in media containing 10% FBS. After formulation, the pH was adjusted, and the colloid solution was pipetted into the donor compartment of the

plate. **A)** Lapatinib and **B)** fulvestrant concentrations in the receiver compartment after 6 h of incubation at 37°C are shown (n=5, one-way ANOVA with Tukey's post-hoc test, ns p>0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SD).

[0039] Figure 21: Most lapatinib that is released at low pH is ionized. Acid-base theory was used to calculate the amount of released lapatinib shown in Figure 12C that is uncharged (n≥9, one-way ANOVA with Tukey's post-hoc test, ***p<0.001, mean ± SD).

[0040] Figure 22: Characterization of transferrin displacement by serum proteins adsorbing to colloidal drug aggregates. Colloids were formulated with 37.5 µM fulvestrant, 12.5 µM lapatinib, and 0.01 mg/mL Alexa Fluor™ 488 labelled transferrin. Measurement of free transferrin revealed that serum proteins displace transferrin from the surface of the colloids (n=3, mean ± SD).

[0041] Figure 23: Trastuzumab-stabilized clotrimazole colloids were disrupted by acid. (A) Colloids of fulvestrant (non-responsive) or clotrimazole (acid-responsive) were formulated at 50 µM and stabilized with 3 µM trastuzumab at pH 7 followed by acidification of pH 5. Scattering intensity drop of clotrimazole colloids is indicative of colloid disruption even when stabilized by trastuzumab. Scattering intensity after acidification indicates that fulvestrant colloids were stable at both pH values over 6 hours while clotrimazole was only stable at pH 7.

[0042] Figure 24: (A) Trastuzumab-stabilized clotrimazole colloids, (B) but not IgG-stabilized colloids, were internalized by HER2-overexpressing SKOV-3 cells. Colloids labeled with cholesteryl BODIPY dye, cell membrane labelled with wheat germ agglutinin and nuclei labeled with Hoechst.

[0043] Figure 25: CACs were measured in pH-adjusted phosphate buffered saline (PBS) by measuring the minimum concentration at which aggregates begin to form using dynamic light scattering. The CAC of A) an imidazole-containing sorafenib analogue is increased with reducing pH whereas the CAC of B) the parent molecule sorafenib is not pH-dependent (n = 3, mean ± 95% CI).

[0044] Figure 26: (A) Graphically depicts the colloid radius as a function of trastuzumab concentration for trastuzimab-stablized colloids of imidazole-containing sorafenib analogue, (B) shows results for colloid preparation in different pH conditions, with or without the trastuzimab stabilizer. The colloids were prepared in different pH conditions, spun down, extracted from the supernatant by organic solvent, dried, reconstituted in DMSO and measured for absorbance.

[0045] Figure 27: Characterization of the cytotoxicity of sorafenib and its acid-responsive analogue (Sorfenib-AL) against cancer cell lines. Cells were incubated for 3 d in media containing different concentrations of drug, and then their viability was assessed by Presto Blue. IC₅₀'s were calculated from the resulting dose-response data using GraphPad Prism 7 (n = 3, mean ± SD).

[0046] Figure 28: Synthetic scheme depicting the synthesis of acid-responsive fulvestrant analogues bearing different tertiary amines. Reagents and conditions: a) dihydropyran (3 equiv), TFA, DCM, rt 3 d; b) PhI(OAc)₂ (3 equiv), ammonium carbamate (4 equiv), methanol, rt, 4 h; c) bromoacetyl bromide, DCM, 0 °C → rt, 30 min; d) nitrogen nucleophile (NuH), or its hydrochloride salt (NuH_xHCl) and DIPEA (5-20 equiv nucleophile), DCM, rt, 3-5 h; e) 2% TFA (>10 equiv) in methanol, rt, 16 h.

[0047] Figure 29. Synthetic scheme depicting the synthesis of acid-responsive fulvestrant analogues bearing different primary amines. Reagents and conditions: a) TBSCl (3 equiv), imidazole (10 equiv), DMF, rt, 16 h; b) PhI(OAc)₂ (3 equiv), ammonium carbamate (4 equiv), methanol, rt, 4 h; c) N-Boc amino acid (glycine or valine) (1.2 equiv), HCTU (1.2 equiv), DIPEA (3.6 equiv), DMF, 0 °C → rt, 16 h; d) acetic acid (5 equiv), TBAF (5 equiv), THF, 60 °C, 4 h; e) DCM (78% v/v), TFA (20% v/v), water (2% v/v), 0 °C, 15 min, rt, 2 h.

[0048] Figure 30. Mass spectrum of an intermediate in the synthesis of acid-responsive fulvestrant analogues demonstrating a major peak and the target mass-to-charge ratio.

[0049] Figure 31. Mass spectrum of an intermediate in the synthesis of acid-responsive fulvestrant analogues demonstrating a major peak and the target mass-to-charge ratio.

[0050] Figure 32. Mass spectrum of an intermediate in the synthesis of acid-responsive fulvestrant analogues demonstrating a major peak and the target mass-to-charge ratio.

[0051] Figure 33. ^1H NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on dimethylamine.

[0052] Figure 34. ^{13}C NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on dimethylamine.

[0053] Figure 35. ESI+ mass spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on dimethylamine.

[0054] Figure 36. ^1H NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on imidazole.

[0055] Figure 37. ^{13}C NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on imidazole.

[0056] Figure 38. ESI+ mass spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on imidazole.

[0057] Figure 39. ^1H NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on morpholine.

[0058] Figure 40. ^{13}C NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on morpholine (DMF used as an internal standard for quantification).

[0059] Figure 41. ESI+ mass spectrum characterizing an acid-responsive fulvestrant analogue bearing a tertiary amine based on morpholine.

[0060] Figure 42. ^1H NMR spectrum characterizing an acid-responsive fulvestrant analogue bearing a primary amine based on glycine.

[0061] Figure 43. ESI+ mass spectrum characterizing an acid-responsive fulvestrant analogue bearing a primary amine based on glycine.

[0062] Figure 44. ESI+ mass spectrum characterizing an acid-responsive fulvestrant analogue bearing a primary amine based on valine.

[0063] Figure 45: Summary of the chemical structures of acid-responsive fulvestrant analogues and measurements of their critical aggregation concentrations (CACs) over a range of pH values. CACs were measured in pH-adjusted phosphate buffered saline (PBS) by measuring the minimum concentration at which aggregates begin to form using dynamic light scattering. The CACs of acid-responsive fulvestrant analogues are increased with reducing pH whereas the CAC of the parent molecule fulvestrant is not pH-dependent ($n = 3-8$, mean $\pm$ SEM).

[0064] Figure 46: CACs were measured in pH-adjusted phosphate buffered saline (PBS) by measuring the minimum concentration at which aggregates begin to form using dynamic light scattering. The CAC of siramesine increases with reducing pH ($n = 4$, mean $\pm$ 95% CI).

[0065] Figure 47: Formulation of siramesine with ultrapure polysorbate 80 (UP80) or bovine serum albumin (BSA) results in stable colloids. Siramesine colloids (500 μ M) were formulated with either UP80 (A) or BSA (B) in phosphate buffered saline. Hydrodynamic diameter was measured over time and remained relatively constant.

[0066] Figure 48: Resuspension of lyophilized siramesine colloids (5 mg/mL) stabilized with bovine serum albumin (BSA, 50 mg/mL) results in relatively monodisperse colloids at high concentrations, as measured by DLS. (A) Size and PDI values indicate the presence of a single population of nanometer-sized colloidal drug aggregates. (B) Scattering intensity measurements indicate that stabilizing excipients enable the resuspension of siramesine colloids after lyophilization.

[0067] Figure 49: Formulation of sorafenib with aDT as an excipient results in stable colloids. Sorafenib colloids (200 μ M) was formulated with 10 or 100 μ g/mL of aDT in phosphate buffered saline (PBS). (A) Hydrodynamic diameter and (B) scattering intensity were measured by dynamic light scattering (DLS) at multiple time points and remained relatively constant over 48 hours.

[0068] Figure 50: Acid-responsive attenuated diphtheria toxin (aDT) stabilizing excipient confers enhanced cytotoxicity of sorafenib colloids to a cancer cell line. SK-OV-3 cells were incubated for 1 h with bovine serum albumin (BSA; 100 µg/mL), aDT (100 µg/mL), BSA-stabilized sorafenib colloids (100 µg/mL BSA, 200 µM sorafenib) or aDT-stabilized sorafenib colloids (100 µg/mL aDT, 200 µM sorafenib) in complete culture media. Cell metabolic activity was determined after 3 days by Presto Blue cell viability reagent and normalized to cells without treatment. aDT-stabilized sorafenib colloids result in the greatest reduction in cell viability.

[0069] Table 1: Colloid-forming drugs with pKa's > 5 show pH-dependent critical aggregation concentrations (CACs) between physiological pH 7.4 and endosomal pH 5.5. CACs were measured in pH-adjusted phosphate buffered saline (PBS) by dynamic light scattering (n = 3, mean ± 95% CI) and pKa's were obtained from DrugBank.ca.

[0070] Table 2: Description of each variable and function used in calculation of the critical aggregation concentration (CAC). This calculation objectively applies a common approach for measuring the CAC where assays are used in which colloidal drug aggregates produce a signal and soluble drug molecules do not. For example, the light scattering intensity of a drug solution does not increase with increasing drug concentration until the CAC is reached, when colloidal particles begin to form. By increasing the concentration above the CAC, the number of colloids increases somewhat linearly, and the scattering intensity increases similarly. This behavior is reflected in Equation S4, which describes a line, the slope of which transitions from zero to positive once the CAC is reached.

[0071] Table 3: Plots of scattering intensity versus concentration that are used to calculate the CAC of each drug at pH 7.4 and 5.5.

[0072] Table 4: Formulations relating treatment name to final concentrations of each component. For imaging and flow cytometry experiments, CholEsteryl BODIPY 542/563 C11 was added before colloid formation to a final concentration of 500 nM.

DETAILED DESCRIPTION

[0073] Definitions

[0074] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0075] As used in the specification and claims, the singular forms “a”, “an” and “the” include plural references unless the context clearly dictates otherwise.

[0076] The term “comprising” as used herein will be understood to mean that the list following is non-exhaustive and may or may not include any other additional suitable items, for example one or more further feature(s), component(s) and/or ingredient(s) as appropriate.

[0077] As used herein, the term “in need thereof” is used to refer to a judgment made by a physician or other caregiver (e.g., a veterinarian) that a subject requires or will benefit from treatment or preventative care. This judgment is made based on a variety of factors that are in the realm of the physician’s or caregiver’s expertise.

[0078] As used herein, “pharmaceutically acceptable” means approved or approvable by a regulatory agency of a federal or a state/provincial government or the corresponding agency in countries other than the United States or Canada, or that is listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, including in humans.

[0079] As used herein, the term “prodrug” will be understood to refer to a compound that, following administration to a subject, is metabolized into a pharmacologically active drug.

[0080] As used herein, the term “subject” refers to a human or a non-human animal (e.g., a mammal).

[0081] The present application provides a colloidal drug aggregate, and related composition, that is designed to enhance the effect of colloid-bound drug against its molecular target by

being responsive to a change in environment as stimulus to trigger drug release from the stable colloids. The change in environment occurs without adding an exogenous reagent by exploiting an endogenous stimulus, such as, for example, the acidic environment of the stomach or of the endosomes and lysosomes of cells. Alternatively, in order to demonstrate this behaviour in the absence of a living organism, the colloidal drug aggregate composition can be triggered to release drug by the addition of an exogenous trigger, such as, for example, an acidic agent or solution.

[0082] The formation of some colloidal drug aggregates is pH-dependent, with the colloidal form of weakly basic drugs dissolving at low pH.¹⁵⁻¹⁷ This behaviour allows colloids that are stable outside the cell to be disrupted intracellularly by the acidic endo-lysosomal pathway.¹⁸⁻²¹ Colloidal drug aggregates stabilized with a targeting antibody have been shown to enter cancer cells via endocytosis,²² such that acidification within the cells can trigger release.

[0083] The present application provides a stable acid-responsive composition comprising a colloidal aggregate comprising one or more drugs, which facilitates acid-triggered release of the drug(s) following administration. Optionally, the stable colloidal aggregate is a targeted composition. The present application further provides methods of manufacture and use of such colloidal drug aggregates. Use of the stable, targeted colloidal drug aggregate-containing composition described herein results in an enhanced effect of the colloid-bound drug against its molecular target over non-colloidal controls and non-acid responsive colloidal controls, due to, for example, colloid endocytosis and subsequent endosomal escape.

[0084] Colloidal drug aggregates

[0085] Many drugs form colloidal drug aggregates in biologically relevant environments, including pharmaceutical excipients, cell culture media and simulated gastrointestinal fluids. Colloidal aggregates have unique nanostructures and are quite different from some other self-assembled drug nanoparticles such as nanocrystals. Colloids typically have a diameter in the range of from about 50 to about 1000 nm and form spontaneously by phase separation on addition to a liquid, such as water or an aqueous solution (the continuous phase). Aggregation is concentration dependent; at low concentrations the compound is fully solubilized and as the concentration increases to a “critical aggregation concentration” (or “CAC”) the

compound spontaneously self-assembles, or aggregates, as a colloid. When the colloid is diluted, the colloid aggregate spontaneously disassembles and the compound returns to its solubilized form. The CAC of a compound is determined by a combination of intrinsic properties of the compound and extrinsic properties, such as, for example, temperature, nature of the continuous phase, and salt concentration. Also, the presence of solubilizing or stabilizing excipients can affect the CAC of a compound.

[0086] Non-limiting examples of drugs that form colloidal drug aggregates include clotrimazole, econazole, miconazole, sulconazole, intraconazole, celecoxib, mefenamic acid, oxaprozin, candesartan cilexetil, manidipine, nicardipine, cinnarizine, glyburide, amiodarone, etravirine, delvirdine, nelfinavir, chlorpromazine, clofazimine, clopidogral sulfate, fenofibrate, meclizine, menatetrenone, pranlukast, raloxifene, triclabendazole, methylene blue, bexarotene, lapatinib, crizotinib, fulvestrant, nilotinib, sorafenib, vemurafenib, gefitinib, imatinib, pazopanib, cabozantinib, and siramesine.

Table: Non-limiting examples of drug compounds known to form colloidal drug aggregates

DRUG	INDICATION/MECHANISM
Clotrimazole	anti-fungal
Econazole	anti-fungal
Miconazole	anti-fungal
Sulconazole	anti-fungal
Intraconazole	anti-fungal
Celecoxib	NSAID
Mefenamic Acid	NSAID
Oxaprozin	NSAID
Candesartan cilexetil	Hypertension
Manidipine	Hypertension
Nicardipine	Hypertension, Ca-channel inhibitor
Cinnarizine	calcium channel blocker
Glyburide	diabetes, K-channel inhibitor
Amiodarone	anti-arrhythmic
Etravirine	anti-HIV, non-nucleoside reverse transcriptase inhibitor

Delvirdine	anti-HIV, non-nucleoside reverse transcriptase inhibitor
Nelfinavir	Antiretroviral, Anti-HIV
Chlorpromazine	anti-psychotic
Clofazimine	anti-bacterial
clopidogral sulfate	anti-platelet/anti-clotting agent
Fenofibrate	hypercholesterolemia
Meclizine	Anti-histamine
Menatetrenone	Hemostatic agent
Pranlukast	anti-asthmatic
Raloxifene	selective estrogen receptor modulator
Triclabendazole	anti-parasitic
Methylene Blue	
Congo Red	
Evans Blue	
Bexarotene	cutaneous T cell lymphoma
Lapatinib	HER2 inhibitor, breast cancer
Crizotinib	ALK inhibitor, non-small cell lung cancer
Fulvestrant	ER antagonist, breast cancer
Nilotinib	Bcr-Abl inhibitor, chronic myelogenous leukemia
Sorafenib	multi-TKI, renal cell carcinoma, hepatocellular carcinoma, thyroid carcinoma
Vemurafenib	B-Raf inhibitor, melanoma
Gefitinib	EGFR inhibitor, non-small cell lung cancer
Imatinib	ABL, c-kit inhibitor, chronic myelogenous leukemia
Pazopanib	multi-kinase inhibitor (VEGFR, PDGFR), renal cell carcinoma
Cabozantinib	c-Met and VEGFR2 inhibitor, thyroid cancer, renal cell carcinoma
Siramesine	Sigma receptor agonist

[0087] In accordance with some embodiments, the colloidal drug aggregate composition of the present application comprises an ionizable drug. For example, the colloidal composition is formed from a colloidal aggregation of one or more ionizable drugs. At least one of the one

or more ionizable drugs is basic, or weakly basic, such that the colloid is susceptible to dissolution in the presence of an acid or acidic environment. In particular, the protonated form of the ionizable drug in the colloidal formulation has a pKa of at least 4.0. Preferably, the pKa of the protonated form of the ionizable drug is at least 4.5, or at least 5.0, or at least 6, or at 6.5. Most dissolved drug molecules are positively charged at pH values below this pKa and uncharged at pH values greater than this pKa.

[0088] Suitable drugs for use in colloid drug aggregates as taught herein are drugs that solubilize in acidic environments and are colloids under physiologically neutral (e.g., approximately pH 7.4 – slightly basic) conditions. An “acidic environment” is defined herein as an environment having a pH of less than 7.4. By ionizing at acidic pH, the drugs in colloids become soluble. The drug colloid aggregate comprising one or more ionizable drug is acid responsive, in that the colloid aggregate will dissolve or disassemble in upon contact with acid or introduction into an acidic environment, thereby releasing the drug from the colloid aggregate. The pH of the acid or acidic environment at which dissolution or disassembly occurs depends on the pKa of the ionizable drug in its protonated form.

[0089] Non-limiting examples of ionizable drugs that can be included in the colloidal drug aggregate composition of the present application include lapatinib, clotrimazole, nilotinib, pazopanib, and siramesine.

[0090] Drugs that form colloidal drug aggregates but that form colloids that do not respond to acid (e.g., sorafenib and fulvestrant), can be chemically modified by addition of an ionizable functional group to form an analogue that is responsive to acid. These drugs are either non-ionizable, or do not ionize in the presence of pH less than 4, until they have been chemically modified to include such an ionizable functional group. These drugs retain activity following chemical modification to include the ionizable functional group, or are metabolized *in vivo* to become biologically/pharmaceutically active. Accordingly, in accordance with some embodiments, the colloidal drug aggregate composition of the present application comprises an ionizable drug analogue that is responsive to acid.

[0091] Drug analogues useful in the formation of the colloidal drug aggregates as described herein, are drug molecules that have been chemically modified to include an ionizable functional group and that, as a result of the chemical modification, have a protonated form

that has a pKa of at least 4.0. Preferably, the pKa of the protonated form of the ionizable drug analogue is at least 4.5, or at least 5.0, or at least 6, or at 6.5.

[0092] Suitable drug analogues for use in colloid drug aggregates as taught herein are drug analogues that solubilize in acidic environments and are colloids under physiologically neutral (e.g., approximately pH 7.4 – slightly basic) conditions. By ionizing at acidic pH, the drug analogues in colloids become soluble. The drug colloid aggregate comprising one or more ionizable drug analogue is acid responsive, in that the colloid aggregate will dissolve or disassemble in upon contact with acid or introduction into an acidic environment, thereby releasing the drug analogue from the colloid aggregate. The pH of the acid or acidic environment at which dissolution or disassembly occurs depends on the pKa of the ionizable drug in its protonated form.

[0093] Non-limiting examples of suitable ionizable functional groups include amines, such as, primary amines, secondary amines, tertiary amines, imines, heterocyclic amines, aromatic amines. More specific examples of suitable amines include, hydroxylamine, hydrazine, imidazole, amine, and triazoles. Alternatively, an aggregator drug can be modified by chemical conjugation to an amine-containing molecule to form an analogue that is responsive to acid. Suitable amine-containing molecules include an amino nitrogen atom which is bound to three other atoms, at least one of which attaches the nitrogen atom to the aggregator molecule through a series of covalent bonds. Optionally, this amino nitrogen atom may be a part of one or more natural amino acids or derivatives thereof, including, but not limited to, β -alanine, N,N-dimethylglycine, N,N-dimethyl- β -alanine, histidine, serine, threonine, asparagine, glutamine, proline, alanine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, glycine, or valine. In this embodiment, the drug analogue exhibits similar activity to the unmodified drug, or the drug analogue functions as a prodrug that is metabolized *in vivo* to form an active drug molecule.

[0094] Non-limiting examples of drug analogues that can be included in the colloidal drug aggregate composition of the present application include a sorafenib analogue (e.g., an imidazole-containing sorafenib analogue), and a fulvestrant analogue (e.g., a glycine or valine modified fulvestrant, or a fulvestrant analogue bearing an amine derived from dimethylamine, imidazole, or morpholine).

[0095] The colloidal formulation additionally comprises a stabilizing agent or excipient. Most colloid-forming drugs aggregate at micromolar or sub-micromolar concentrations. Colloids formed without stabilizing excipients are often polydisperse and precipitate over several hours in physiological media, are unstable and, therefore, unsuitable for use according to the present invention. Excipients, including polymers, proteins, and other colloid-forming compounds, such as other drugs or azo-dyes, can control the size and stability of colloidal drug aggregates. In addition to stabilizing colloidal drug aggregates, proteins (e.g., antibodies) can also confer functionality to promote selective uptake by target cells.

[0096] In certain embodiments, the colloidal drug aggregate of the present application comprises a corona. As used herein, the term “corona” refers a surface-bound layer of adsorbed proteins and/or other stabilizing excipient(s), onto a colloidal aggregate, for example, resulting from the exposure of the aggregate to solution and media containing proteins and/or other stabilizing excipient(s). In one embodiment, in which the conditions for concentration and nature of the proteins and/or excipient(s) in the solution and media is known, the corona may be well defined and characterized. The corona formation and its characteristics may be influenced by various factors such as the surface chemistry of the nanoparticle, the colloidal aggregate size, and the composition of the continuous phase.

[0097] Proteins that can be used for the protein corona may be, but are not limited to, albumins, immunoglobulins, caseins, insulins, hemoglobins, lysozymes, α -2-macroglobulin, fibronectins, vitronectins, fibrinogens, lipases, transferrin, apolipoproteins, bacterial toxins, and the like. Proteins, peptides, enzymes, antibodies and combinations thereof, may also be used to stabilize the colloidal aggregates. According to an embodiment, the use of novel antibodies or FDA-approved antibodies such as tocilizumab, natalizumab, vedolizumab, alemtuzumab, cetuximab, daratumumab, ofatumumab, panitumumab, pertuzumab, trastuzumab, dinutuximab, obinutuzumab, ramucirumab, ipilimumab, nivolumab, pembrolizumab, brentuximab, catumaxomab, basilixumab, ibritomumab may be tailored to stabilize the colloidal aggregates. The target/indication for these FDA approved antibodies are provided in the table below.

Table: Non-limiting examples of antibody therapeutics that have been approved by FDA

ANTIBODY	TARGET/INDICATION
Tocilizumab	IL-6R, rheumatoid arthritis
Natalizumab	α 4-integrin, Crohn's disease, multiple sclerosis
Vedolizumab	α 4b7-integrin, Crohn's disease, ulcerative colitis
Alemtuzumab	anti-CD52, chronic lymphocytic leukemia, cutaneous T-cell lymphoma, multiple sclerosis
Cetuximab	EGFR, colorectal cancer, head and neck cancer
daratumumab	CD38, multiple myeloma
ofatumumab	CD20, chronic lymphocytic leukemia
Panitumumab	EGFR, colorectal cancer
Pertuzumab	HER2, breast cancer
Trastuzumab	HER2, breast cancer,
Dinutuximab	GD2, neuroblastoma
obinutuzumab	CD20, chronic lymphocytic leukemia
Ramucirumab	VEGFR2, non-small cell lung cancer, gastric/gastroesophageal junction adenocarcinoma
Ipilimumab	CTLA-4, melanoma
Nivolumab	PD-1, melanoma
Pembrolizumab	PD-1, melanoma, NSCLC,
Brentuximab	CD20, ADC with monomethyl auristatin E for chronic lymphocytic leukemia
Catumaxomab	anti-EpCAM/anti-CD3, malignant ascites
Basilixumab	IL-2R alpha chain, immunosuppression
Ibritumumab	CD20, conjugated to radioisotope chelator for non-hodgkin's lymphoma

[0098] In accordance with other embodiments, the acid-responsive colloidal drug composition comprises a combination of one or more non-ionizable, or ionizable, drugs with

an acid-responsive stabilizer. This acid-responsive stabilizer acts to disrupt the colloid or cellular membranes in an acidic environment. In particular, an acid-responsive stabilizing agent is one that undergoes a morphological and/or functional change when the pH is reduced to less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4.

[0099] Non-limiting examples of acid-responsive stabilizers include bacterial toxins, such as attenuated diphtheria toxin or variants thereof.

[00100] Accordingly, certain embodiments of the present application provide colloidal drug aggregate compositions that are useful in targeting a drug for treatment of a disease or disorder in a subject. In specific embodiments, the disease or disorder is selected from the diseases and disorders associated with the listed FDA-approved antibodies, as set out in the table above. This list is not exhaustive; therapeutic antibodies and targeting antibodies continue to be developed and can be incorporated in the colloidal drug aggregate compositions of the present application as a stabilizing agent, a targeting agent, or both.

[00101] In other embodiments, the colloidal drug aggregate composition of the present application comprises a stabilizing agent that is another aggregator drug, which may or may not be acid responsive. In a particular embodiment, the colloidal drug aggregate composition comprises an acid responsive (i.e., ionizable) drug and a non-acid responsive drug. In one example of this embodiment, the acid responsive drug is lapatinib and the non-acid responsive drug is fulvestrant.

[00102] Colloidal drug aggregate formulations of the present application can be formulated simply by dissolving a drug or drug analogue in a water-miscible solvent (e.g., DMSO) and adding water, or an aqueous solution, to the dissolved drug and allowing the spontaneous formation of colloids. One or more stabilizing agents are incorporated into either aqueous or organic phase prior to colloid formation, or to the mixture after colloid formation. For convenience, the formation of the colloidal drug aggregate is often done at ambient temperature, typically without the need for further mixing or stirring. The amount of the drug used will depend on the CAC for that drug under the formulation conditions used. Colloids are usually detectable within seconds of mixing the drug, or drug analogue, in the water-miscible solvent with the aqueous phase. In accordance with certain embodiments in which

the colloidal drug aggregate formulation is for pharmaceutical use, the one or more stabilizing agents are cytocompatible. Also, as noted above, in certain embodiments the one or more stabilizing agents are acid-responsive.

[00103] In some instances, the pH of the colloid solution needs to be adjusted to facilitate colloid formation or for colloid stability. The pH can be adjusted using an adjusting agent that is an acid (e.g., HCl or citric acid) or a base (e.g. NaOH), as necessary. pH adjustment is typically performed by adding the pH adjusting agent as the final formulation step. Depending on the amount of pH adjusting agent required, it may be necessary to reduce the amount of water, or aqueous solution, used in the colloid formation.

[00104] Pharmaceutical formulations and use thereof

[00105] The present application further provides pharmaceutical formulations for administration of a colloidal drug aggregate composition to a subject in need thereof. The pharmaceutical formulation of the present application comprises a colloidal drug aggregate, optionally in combination with a pharmaceutically acceptable carrier, diluent, excipient or medium, wherein the colloidal drug aggregate comprises one or more drugs and a stabilizing agent, and wherein the colloidal aggregate disrupts, dissolves or disassembles when the acid-responsive composition is in an acid environment.

[00106] In certain embodiments, the pharmaceutical composition comprises a combination of two or more colloidal drug aggregate compositions.

[00107] Suitable pharmaceutical compositions of the present application will generally include an amount of the one or more drugs to give a range of final concentrations, depending on the intended use.

[00108] In certain embodiments, the pharmaceutical compositions of the present application comprise a colloidal drug aggregate composition as the sole active component, alone or in combination with one or more desired pharmaceutically inactive additives, excipients, and/or components (e.g., polymers, fillers, carriers, excipients, diluents, disintegrating additives, lubricants, solvents, dispersants, absorption promoting additives,

controlled release additives, anti-microbial additives, preservatives, sweetening additives, colorants, flavors, dyes, or the like), and no other active pharmaceutical ingredient(s).

[00109] In other embodiments, the pharmaceutical composition comprises a combination of two or more colloidal drug aggregate compositions as the active components, alone or in combination with one or more desired pharmaceutically inactive components as listed above.

[00110] In alternative embodiments, the pharmaceutical composition comprises one or more colloidal drug aggregate compositions in combination with one or more other therapeutic agent.

[00111] Accordingly, the present application further comprises a method of providing drug therapy to a subject by administering a pharmaceutical composition as described above. Such a method is particularly useful for delivering or targeting the one or more drugs in the pharmaceutical composition to an acidic target site of the patient (e.g., stomach, lysosomes, endosomes, etc.).

[00112] In a specific embodiment, the pharmaceutical composition comprises a colloidal drug aggregate composition comprising lapatinib. Accordingly, the present application further comprises a method of treating cancer, such as breast cancer, comprising administration of a colloidal drug aggregate composition comprising lapatinib to a subject in need thereof. Optionally, the method additionally comprises administration of another chemotherapeutic agent before, after or simultaneous with administration of the colloidal drug aggregate composition comprising lapatinib.

[00113] In another specific embodiment, the pharmaceutical composition comprises a colloidal drug aggregate composition comprising lapatinib and fulvestrant. Accordingly, the present application further comprises a method of treating cancer, such as breast cancer, comprising administration of a colloidal drug aggregate composition comprising lapatinib and fulvestrant to a subject in need thereof. Optionally, the method additionally comprises administration of another chemotherapeutic agent before, after or simultaneous with administration of the colloidal drug aggregate composition comprising lapatinib and fulvestrant.

[00114] In another specific embodiment, the pharmaceutical composition comprises a colloidal drug aggregate composition comprising clotrimazole. Accordingly, the present application further comprises a method of treating a fungal infection, such as a fungal skin infection, comprising administration of a colloidal drug aggregate composition comprising clotrimazole to a subject in need thereof. Optionally, the method additionally comprises administration of another chemotherapeutic agent before, after or simultaneous with administration of the colloidal drug aggregate composition comprising clotrimazole.

[00115] In another specific embodiment, the pharmaceutical composition comprises a colloidal drug aggregate composition comprising sorafenib or an analogue of sorafenib. Accordingly, the present application further comprises a method of treating cancer, such as kidney cancer, liver cancer, or thyroid cancer, comprising administration of a colloidal drug aggregate composition comprising the sorafenib analogue to a subject in need thereof. Optionally, the method additionally comprises administration of another chemotherapeutic agent before, after or simultaneous with administration of the colloidal drug aggregate composition comprising the sorafenib analogue.

[00116] In another specific embodiment, the pharmaceutical composition comprises a colloidal drug aggregate composition comprising an analogue of fulvestrant. Accordingly, the present application further comprises a method of treating cancer, such as breast cancer, comprising administration of a colloidal drug aggregate composition comprising the fulvestrant analogue to a subject in need thereof. Optionally, the method additionally comprises administration of another chemotherapeutic agent before, after or simultaneous with administration of the colloidal drug aggregate composition comprising the fulvestrant analogue.

[00117] To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

[00118] EXAMPLE 1: Colloidal Lapatinib Aggregate Preparation and Use

[00119] Methods

[00120] *Materials*

[00121] Lapatinib and sorafenib were purchased from MedChemExpress. Nilotinib and pazopanib were purchased from Cedarlane. Fulvestrant was purchased from SelleckChem. Poly(D,L-lactide-co-2-methyl-2-carboxy-trimethylene carbonate)-graft-poly(ethylene glycol) (PLAC-PEG) was synthesized by ring-opening polymerization using a pyrenebutanol initiator to a molecular weight of 12 kDa and conjugated with an average of three 10 kDa PEG chains/backbone as previously described.⁴³ Ultrapure polysorbate 80 (UP80) was purchased from NOF America Corporation. Clotrimazole, norethindrone, Dimethyl sulfoxide (DMSO), transferrin, EDTA, dodecane, lecithin, polyacrylic acid (PAA), methylcellulose, and hydroxypropylmethylcellulose (HPMC) were purchased from Sigma-Aldrich. Transferrin-Alexa Fluor 488 conjugate, RPMI 1640 cell culture medium, penicillin-streptomycin solution, trypsin-EDTA solution, Hank's balanced salt solution, PrestoBlue cell viability reagent, CholEsteryl BODIPY™ 542/563 C11, CellMask Green™ 1000x solution, and 7-AAD were purchased from Thermo Fisher Scientific. Fetal bovine serum and Dulbecco's phosphate buffered saline were purchased from Wisent Bio Products. Ultrapure Congo red was purchased from Enzo Life Sciences. HPLC grade acetonitrile and methanol were purchased from Caledon Laboratories. Mass spectrometry grade formic acid was purchased from Fluka.

[00122] *Calculation of the critical aggregation concentration (CAC)*

[00123] CAC values are calculated by preparing colloidal formulations with different concentrations and measuring the scattering intensity (or fluorescence intensity when appropriate). The data is then fit to Equation S4, which was derived from first principles (Equations S1–S3), using the MATLAB curve fitting toolbox to obtain the critical aggregation concentration (CAC) under the conditions used. Table 2 describes the meaning of each variable; Figure 1 shows the result of curve-fitting this model to experimental data.

$$Signal = \sum_i Signal_i = Signal_{BL} + Signal_{colloids} \quad S1$$

Assuming constant colloid diameter:

$$Signal_{colloids} \propto C_{agg,colloids} \quad S2$$

$$C_{agg,colloids} = \begin{cases} 0, & C_{agg} < CAC_{agg} \\ C_{agg} - CAC_{agg}, & C_{agg} \geq CAC_{agg} \end{cases} = H(C_{agg} - CAC_{agg}) \cdot (C_{agg} - CAC_{agg}) \quad S3$$

Substituting and adding a qualitative correction factor for diameter variations:

$$Signal = Signal_{BL} + m \cdot H(C_{agg} - CAC_{agg}) \cdot (C_{agg} - CAC_{agg})^n \quad S4$$

[00124] *Colloidal drug aggregate formulation*

[00125] Colloidal drug aggregates were formulated as described previously.⁸ Briefly, the colloids formed spontaneously when water was added to drug that was dissolved in a water-miscible organic solvent (usually DMSO). Final colloid suspensions were typically made at a 1 mL scale with 1% (v/v) final DMSO concentration. First, solutions of drug, polymer, and dye were prepared in 10 μ L DMSO at 100 $\times$ the final concentration. For colloids formulated under serum-free conditions, 890 μ L of double distilled water was then added, followed by 100 μ L of 10 $\times$ PBS (for experiments without cells) or 10 $\times$ RPMI 1640 (for cell experiments). For colloids formulated under 10% (v/v) serum conditions, 800 μ L of double distilled water was added, followed by 90 μ L of 10 $\times$ PBS or 10 $\times$ media, and finally 100 μ L of FBS. Transferrin stabilized colloids were prepared by supplementing the water added with a small amount of 5 mg mL⁻¹ transferrin in PBS. When the pH of the colloid solution needed to be adjusted, the amount of water added was reduced by 10 μ L, and 10 μ L of aqueous citric acid was added as the last formulation step. The concentration of citric acid used depended on the desired final pH. In PBS, the concentration was 0.12 M for pH 6.5, 0.3 M for pH 5.5, 0.35 M for pH 5.2, and 0.5 M for pH 4.5. In PBS with 10% (v/v) FBS, the concentration was 0.15 M for pH 6.5, 0.35 M for pH 5.5, and 0.6 M for pH 4.5. For time-based studies, the colloid suspensions were incubated at 37°C between time points.

[00126] *Characterization by dynamic light scattering*

[00127] Colloid diameter, polydispersity index (dispersity), and scattering intensity were measured by dynamic light scattering (DLS) using a DynaPro Plate Reader II (Wyatt Technologies) that was optimized by the manufacturer for detection of colloidal aggregates (i.e. 100-1000 nm particles). The instrument was configured with a 60 mW 830 nm laser and detector angle of 158°. A 100 µL sample of each formulation was pipetted into a 96-well plate and measured with 20 acquisitions per sample.

[00128] *Characterization of zeta potential*

[00129] Colloids were prepared as described above, but with a single addition of 0.1 mM KCl solution instead of water and buffer. Zeta potential was immediately assessed using a Malvern Nano-ZS (Malvern Panalytical).

[00130] *Fluorescent intensity characterization*

[00131] Colloid suspensions were prepared as described above. A 100 µL sample of each formulation was pipetted into a 96 well plate. Fluorescence was measured using a Tecan Infinite Pro 200 plate reader.

[00132] *Characterization by transmission electron microscopy (TEM)*

[00133] Colloidal formulations (5 µL) were deposited onto freshly glow-discharged 400 mesh carbon coated copper TEM grids (Ted Pella, Inc.) and allowed to adhere for 5 min. Excess liquid was removed, followed by a quick wash with 5 µL water. Grids were then imaged on a LEO 912B Energy Filtered TEM operating at 120 kV.

[00134] *Assessment of colloid-bound transferrin*

[00135] Colloid formulations were prepared as described above with transferrin-Alexa Fluor 488 conjugate as the stabilizer. At predetermined time points during incubation at 37 °C, formulations were centrifuged at 16,000×g for 1 h, followed by withdrawal of 100 µL of supernatant for measurement. The amount of transferrin remaining in the supernatant was measured using fluorimetry ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$). The fraction of transferrin bound to the colloid was calculated with Equation 1.

$$f_{bound} = 1 - f_{free} = 1 - \frac{I_{colloid+Tf488}}{I_{Tf488}} \quad 1$$

[00136] *Assessment of drug release*

[00137] Drug release was measured by centrifuging to pellet the colloids, and then by quantifying the drug in the supernatant. Colloid formulations were centrifuged at 16,000×g for 1 h, followed by withdrawal of 100 µL for quantification. Another 100 µL was withdrawn for DLS analysis to confirm that the colloids had completely settled out of solution. Non-centrifuged colloid suspensions were used as controls.

[00138] *Membrane permeability assay*

[00139] Supported artificial lipid membranes were prepared according to the manufacturer instructions (EMD Millipore cat# MATRNPS50). Briefly, a 1% (w/w) solution of egg lecithin in dodecane was prepared by sonicating until the solution was no longer cloudy. A 5 µL aliquot of this phospholipid solution was dropped by pipette into each well of the mesh plate, which wet the membrane and caused it to become translucent. Receiver solution was prepared as 1% (v/v) DMSO and 10% (v/v) FBS in PBS and 340 µL was pipetted into each well of the receiver plate. Lapatinib and fulvestrant solutions ranging from 0 to 5 µM for constructing the standard curve were prepared in identical media and pipetted into separate wells of the receiver plate. Colloids were prepared in PBS containing 10% (v/v) FBS as described above, and 150 µL was pipetted into the mesh plate wells corresponding to the receiver solution in the receiver plate. The mesh plate and receiver plate were gently mated, resulting in the receiver solution wetting the bottom of the phospholipid membrane. A plate cover was added, and the assembly edges sealed with parafilm to prevent evaporation. The sealed assembly was then incubated at 37°C for 6 h. Finally, the stack was carefully disassembled and a 200 µL sample of receiver and standard curve solutions was withdrawn for drug quantification as described below. Each donor and receiver well were tested with a pH strip to verify integrity of the membrane. A separate test with Trypan blue verified that the membranes were impermeable to this colloidal dye.

[00140] *Drug concentration quantification*

[00141] Drug concentration in samples from drug release and membrane permeability assays was quantified by high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-MS-MS). Protein was precipitated from samples in serum containing media by spiking with 10 μ L of formic acid and adding acetonitrile to a final volume of 1 mL. The precipitated samples were then centrifuged at 16,000 $\times$ g for 5 min to pellet the proteins. The drug-containing supernatant was then diluted in methanol such that the final drug concentration was less than 100 ng mL⁻¹. During the final dilution, internal standards (nilotinib for lapatinib and norethindrone for fulvestrant) were added to a final concentration of 25 ng/mL each. Standard curves were prepared in a similar way, by diluting 100 mM solutions of drug in DMSO with methanol to final concentrations of 100, 75, 50, 25, 10, 5, 2.5, and 1 ng mL⁻¹, each with 25 ng mL⁻¹ of internal standard.

[00142] *Cell culture*

[00143] MDA-MB-231-H2N cells were a generous gift from Dr. Robert Kerbel (Sunnybrook Research Institute, Toronto, ON, Canada). Cells were maintained in a humidified incubator at 37°C with 5% atmospheric CO₂. Cells were grown in 75 cm² tissue culture flasks with 10 mL RPMI 1640 supplemented with 10% FBS, 10 UI/mL penicillin, and 10 μ g mL⁻¹ streptomycin. Cells were passaged twice per week with typical subculture ratio of 1:16.

[00144] *Cell viability experiments*

[00145] After passaging, cell suspensions were diluted into fresh media and 200 μ L was pipetted into each well of a 96 well plate. Two thousand cells per well were plated and allowed to adhere overnight. Then, the media was withdrawn and replaced with treatment formulations (prepared as described above). Cells were incubated during the experiment in a humidified incubator at 37°C with 5% atmospheric CO₂. For treatments lasting less than 3 d, the treatment solutions were removed after the prescribed time, cells were washed with fresh media, and 200 μ L of fresh media was added. Cell viability was assessed 3 days after commencing treatment using the PrestoBlue™ viability assay according to the

manufacturer's protocol. Cell viability was reported as a percentage of the vehicle (DMSO with no drug or excipient) control.

[00146] *Confocal imaging of treated cells*

[00147] Cells were seeded at approximately 2×10^5 cells per well in 8-chamber tissue culture treated glass cover slips and allowed to adhere overnight. Treatments were prepared as described above and 300 μL applied to each well. Cells were incubated during the experiment in a humidified incubator at 37°C with 5% (v/v) atmospheric CO_2 . The treatment solutions were removed after the prescribed time, cells washed with fresh media, and 300 μL of fresh media added. For fixed cells, 4% (w/w) aqueous paraformaldehyde was applied for 10 min, followed by staining (when appropriate), and finally blank PBS. For live cell conditions, cells were washed with fresh media, stained, and imaged under Hank's balanced salt solution. Cells were imaged on an Olympus FV1000 inverted confocal microscope using a 1.42 N.A. 60x oil immersion lens (Olympus PLAPON 60XO). Laser and detector settings were held constant between different treatment conditions.

[00148] *Flow cytometry*

[00149] Cells were seeded at approximately 2×10^5 cells per well in 24-well plates and allowed to adhere overnight. Treatments were prepared as described above and 500 μL was applied to each well. Cells were incubated during the experiment in a humidified incubator at 37°C with 5% (v/v) atmospheric CO_2 . For treatments lasting less than 3 h, the treatment solutions were removed after the prescribed time, cells were washed with fresh media, and 500 μL of fresh media was added. Three hours after treatment initiation, cells were washed three times with media, detached using 500 μL of accutase solution, spiked with 500 μL of media, then centrifuged at $400\times g$ to pellet the cells. Cells were then resuspended in cold flow buffer (PBS supplemented with 2% (v/v) FBS and 2 mM EDTA) and kept on ice until measurement. The flow buffer was supplemented with 7-AAD at $2 \mu\text{g mL}^{-1}$ as a vital stain, except for blank controls and in the endosome escape assay where 7-AAD had already been added. Cell fluorescence was quantified using a BD Accuri™ C6 flow cytometer with excitation wavelength of 488 nm and emission filters of 585/40 nm (BODIPY colloid dye) and >670 nm (7-AAD). Data were analyzed using the BD Accuri™ C6 Plus software and

reported as the fluorescence of the live cell fraction (gated using scattering and 7-AAD) averaged between three biological replicates.

[00150] Results

[00151] This study was initiated by identifying colloid-forming drugs that could respond to acidic conditions by measuring the critical aggregation concentration (CAC) of several aggregators as a function of pH (Table 1). Critical aggregation concentrations were calculated by plotting the scattering intensity (indicative of colloid number and size) from dynamic light scattering (DLS) versus concentration (Table 2, Figure 1, Table 3).^{1,23} Weakly basic aggregators, such as clotrimazole and lapatinib, had pH-dependent CACs, which enable colloid disruption as a function of pH; conversely, aggregators with pKa values well below the identified pH range, such as fulvestrant and sorafenib, did not dissolve on acidification.

[00152] A stable colloidal formulation of lapatinib was developed due to its potency and robust response to acidic pH. Although lapatinib forms colloidal aggregates on its own, they are only transiently stable and precipitate within a few hours (Figure 2). Excipients were screened to stabilize colloidal lapatinib against precipitation (Figure 2, Figure 3)^{8,24-26} and it was found that co-aggregation with fulvestrant, another colloid-forming drug, resulted in stable, spherical colloids, approximately 200 nm diameter (Figure 4) with slight positive charge (Figure 5). It was also found that a 1:3 molar ratio of lapatinib:fulvestrant was best stabilized against precipitation by excipients such as the graft copolymer poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (PLAC-PEG, Figure 6, Figure 7) and the protein transferrin (Figure 8, Figure 9). It was also confirmed that these colloids were stable in cell culture media (Figure 10).

[00153] The response of the stable colloids to acidic conditions was investigated. Colloids were formulated in PBS and subsequently acidified, mimicking the pH within the endo-lysosomal pathway. To determine whether the lapatinib-fulvestrant co-colloids changed as a function of pH, colloid size and scattering intensity were measured by DLS (Figure 11). Relatively little change in colloid size after acidification was observed. Next, the suspension was centrifuged to pellet the colloids, and then free drug in the supernatant was quantified after ensuring that no colloids were present. The amount of released lapatinib increased with increasing acidity (Figure 12A) whereas the release of the non-ionizable fulvestrant was

unaffected by pH (Figure 12B). Importantly, lapatinib release could be triggered at any time after formulation, and rapidly reached an equilibrium that was sustained for 24 h (Figure 12C) whereas fulvestrant remained unresponsive to acid (Figure 12D). The amount of drug released from the colloids remained similar from both 50 μ M to 200 μ M colloidal aggregates (Figure 13). Additionally, similar trends were observed (Figure 14) in PBS containing 10% (v/v) fetal bovine serum (FBS), demonstrating a similar response in serum-containing media. An increased baseline level of drug release in serum-containing media was also observed, which is consistent with previous reports that show how proteins increase the CAC of colloidal aggregators.^{10,18} These results demonstrate that the stable lapatinib colloids will respond to the acid stimulus in the endo-lysosomal pathway.

[00154] The endocytosis of the stable, acid-responsive colloidal drug aggregates was investigated. To measure uptake, a hydrophobically-modified BODIPY dye, which is fluorescent only when incorporated within the colloid,²² was incorporated. These colloids appeared as punctate structures within MDA-MB-231-H2N cells (Figure 15, Figure 16). Although minimal uptake of the PLAC-PEG stabilized colloids observed, it was found that transferrin-stabilized colloids were taken up to a much greater extent (Figure 3C). This result held for both lapatinib/fulvestrant colloids and colloids of fulvestrant alone, and aligns with previous research demonstrating that stabilizers that interact specifically with cells can elicit endocytosis.²²

[00155] The *in vitro* toxicity of acid-responsive, stable lapatinib/fulvestrant colloids was then examined against lapatinib-sensitive, HER2-overexpressing MDA-MB-231-H2N cells. The transferrin-stabilized lapatinib/fulvestrant colloids were substantially more toxic than any other formulation, including PLAC-PEG stabilized lapatinib/fulvestrant colloids that were minimally endocytosed (Figure 17A).

[00156] To determine the mechanism of the increased toxicity of endocytosed lapatinib-containing colloids versus non-endocytosed colloids, the ability of colloidal lapatinib to disrupt the endosomes was investigated using a fluorescence dequenching assay.^{29,30} Incorporating the membrane impermeant nuclear stain, 7-aminoactinomycin D (7-AAD), into the colloids allowed measurement of its fluorescence in MDA-MB-231-H2N cells. The fluorescence of 7-AAD intensifies on DNA binding. Therefore, 7-AAD staining

should only be observed in the nucleus following both colloid endocytosis and subsequent endosomal escape. Lapatinib/fulvestrant-transferrin colloids resulted in higher nuclear 7-AAD fluorescence than fulvestrant-transferrin colloids and PLAC-PEG stabilized formulations (Figure 17B-C, Figure 18). Viability of the cells remained unchanged after 3 h of treatment, indicating that drug toxicity did not interfere with this assay (Figure 19).

[00157] An alternative explanation to endosome disruption is simple diffusion of lapatinib from the endosomes, which was probed with a membrane permeability assay. The transmembrane diffusion of lapatinib was reduced when the colloids were acidified (Figure 20), which, without wishing to be bound by theory, was likely because the concentration of free, uncharged lapatinib decreased with increasing acidity (Figure 21).

[00158] Notwithstanding the importance of transferrin for cellular uptake, its stability in serum was investigated since displacement of the macromolecular coronas by serum proteins can affect circulating nanoparticles.²⁷ To test whether serum proteins could displace the transferrin stabilizer, colloidal drug aggregates stabilized with fluorophore-labelled transferrin were formulated and then pelleted by centrifugation. Quantifying the fraction of transferrin remaining in the supernatant, it was found that most of the transferrin was displaced after 6 hours of incubation in 10% serum (Figure 22).

[00159] Discussion

[00160] This study demonstrates that it is possible to trigger the release of weakly basic colloid-forming drugs from stable colloidal drug aggregates by acidification of the medium. By exploiting the local acidity in the endo-lysosomal pathway, drug release and its consequent cytotoxicity was triggered, thereby overcoming a key challenge of colloidal stability, and hence inactivity, after cell uptake. Thus, whereas previous research has shown that colloidal aggregation can cause false negative hits in cytotoxicity assays,^{11,12} this effect can be overcome by protonation of the colloidal drug and subsequent endosome disruption.

[00161] Interestingly, it was found that the amount of lapatinib released was less than its CAC at a comparable pH. Without wishing to be bound by theory, this behaviour may be attributed to the fulvestrant, which remains in the colloidal state and acts as a sink for lapatinib. This hypothesis is supported by studies on the phase behaviour of co-colloids that

show a reduction in effective CAC when multiple drugs co-exist in the colloid.³¹⁻³³ This physical interaction between lapatinib and fulvestrant suggests that they mix to form co-colloids and may explain how fulvestrant stabilized lapatinib against precipitation.

[00162] It was found that endocytosis of the acid-responsive lapatinib-fulvestrant colloids greatly enhanced their cytotoxicity due to increased drug transport from the endosomes into the cytosol. This endosomal escape could occur by one of two mechanisms: either the increased concentration of free lapatinib enhances the amount of lapatinib diffusion across the endosomal membrane or the weakly basic lapatinib disrupts the integrity of the endosomes through osmotic pressure effects.^{29,34,35} As lapatinib is slow to cross membranes under acidic conditions, the enhanced diffusion mechanism is unlikely. Transport of ionizable drugs, such as protonated (cationic) lapatinib at reduced pH, diffuse more slowly through the lipid membranes.^{36,37} This prediction was supported by the estimate of free, uncharged lapatinib as a function of pH. With respect to the proton-sponge mechanism, the 7-AAD fluorescence quenching assay showed that acid-responsive colloidal drug aggregates can disrupt endosomes, leading to drug leakage into the cytosol and ultimately enhancing drug cytotoxicity. This type of endosomal escape strategy has been exploited to deliver therapeutics that are unable to escape the endosomes themselves.^{35,38,39} This approach has been adapted here and has been surprisingly found to effectively deliver a new type of cargo: colloidal drug aggregates. Furthermore, the approach employed here successfully avoided the use of acid-responsive but pharmacologically inert excipients by using a drug that is naturally acid-responsive. Such pharmacologically active drug colloids can be used instead of the traditionally used inert carriers to deliver proteins or nucleic acids.

[00163] Notwithstanding these results, colloidal drug aggregates are admittedly in dynamic equilibrium with free drug, and a small amount of that free drug may diffuse across lipid membranes. As a result, cells that are highly sensitive to lapatinib may be killed even in the absence of colloid endocytosis. Another consequence of this behaviour is that drug diffusion out of the endo-lysosomal pathway could slowly occur simultaneously with the proton sponge effect.

[00164] The present study demonstrates that endosomal escape of lapatinib is enhanced by the acidic microenvironment. Importantly, endosomal escape in live cells was

shown to occur through a membrane disruption mechanism. Furthermore, it was shown that only colloidal drugs that are inherently acid-responsive can have their release triggered by acidic conditions whereas those that are unresponsive to acid cannot.

[00165] Conclusions

[00166] Recent attempts to exploit colloidal drug aggregates have been, hindered, until now, by the inability to control drug release. The present study showed acid-triggered release from stable colloidal drug aggregates, endosomal disruption, and enhanced cytotoxicity. The selective, stimulus-responsive release of drugs from colloidal aggregates was demonstrated. This provides demonstration of controlled release of a therapeutic molecule from a colloid that is inactive until its target is reached.

[00167] EXAMPLE 2: Trastuzumab-stabilized clotrimazole colloids

[00168] Methods

[00169] *Materials*

[00170] Fulvestrant was purchased from Selleck Chemicals. Clotrimazole, IgG from human serum, and RPMI 1640 cell culture media were purchased from Sigma-Aldrich. Trastuzumab was obtained from Roche. CholEsteryl BODIPY 542/563 C11, Hoechst 33342, and wheat germ agglutinin Alexa Fluor 647 conjugate were purchased from Thermo Fisher scientific. SKOV3 cells were purchased from ATCC. Fetal bovine serum was purchase from Wisent Bio Products.

[00171] *Colloid formulation and characterization by dynamic light scattering*

[00172] Colloidal drug aggregates were formulated as described as described in Example 1 using clotrimazole or fulvestrant in place of lapatinib. In particular, colloids of fulvestrant (non-responsive) or clotrimazole (acid-responsive) were formulated at 50 μ M and stabilized with 3 μ M trastuzumab at pH 7 followed by acidification of pH 5. These formulations were assessed by dynamic light scattering as described in Example 1.

[00173] *Cell culture and confocal microscopy*

[00174] Cell culture was carried out as described in Example 1. SKOV3 cells were seeded at 10,000 per well in 16-well glass chamber slides. Fluorescent clotrimazole colloids were prepared as described above, with the addition of 500 nM CholEsteryl BODIPY 542/563 C11. Trastuzumab or IgG were added to a final concentration of 3.5 μ M, the formulations were incubated for 10 minutes, and then FBS was added to a final concentration of 5% (v/v). The cell media was replaced with the colloid formulations and incubated for 3 h. The formulations were removed and the cells were fixed with 4% paraformaldehyde. Following fixation, wheat germ agglutininin Alefa Fluor 647 conjugate was added to stain the cell membranes according to the manufacturer protocol and counterstained with Hoechst. Cells were then imaged on an Olympus FV1000 confocal microscope at 60 $\times$ magnification.

[00175] Results

[00176] As shown in Figure 23 scattering intensity after acidification indicates that fulvestrant colloids were stable at both pH values (pH 7 and pH 5) over 6 hours while clotrimazole is only stable at pH 7. This indicates that the clotrimazole-containing colloidal drug aggregate was acid-responsive, while the fulvestrant-containing colloidal drug aggregate was not acid-responsive.

[00177] Figure 24 demonstrates that the trastuzumab-stabilized clotrimazole colloids, but not IgG-stabilized colloids, are internalized by HER2-overexpressing SKOV-3 cells.

[00178] Discussion

[00179] These results demonstrated that the colloid-forming compound clotrimazole is acid-responsive, and that colloids of clotrimazole can target cancer cells by coating the colloids with targeting antibodies.

[00180] EXAMPLE 3: Sorafenib analogue-containing colloids

[00181] Methods

[00182] *Materials*

[00183] A sorafenib analogue containing a pendant imidazole functional group on the N-methylpyridine-2-carboximide was synthesized to specifications by Enamine. Phosphate buffered saline was purchased from Wisent Bio Products. Hydrochloric acid was purchased from VWR. Cell lines were obtained as a generous gift from R. Kerbel (MDA-MB-231-H2N) or from ATCC (BT474, SKBR3, SKOV3).

[00184] *Colloid formulation and CAC measurement*

[00185] Colloids were formulated as described in Example 1. Briefly, solutions of sorafenib analogue in DMSO were prepared at various concentrations and then diluted 100-fold with PBS at different concentrations. 100 μ L of each sample was analyzed by DLS and the CAC's calculated as described in Example 1.

[00186] *Cell culture and IC₅₀ determination*

[00187] Cell culture and experiments were carried out as described in Example 1. IC₅₀'s were calculated using GraphPad Prism 7 from cell viabilities measured after treating with different concentrations of drug for 3 days.

[00188] Results

[00189] Figure 25 shows a comparison of the CACs for sorafenib and the sorafenib analogue over a range of pH values. The increase in CAC values found for the sorafenib analogue as the pH decreases is indicative of the acid responsiveness of the colloidal drug aggregate comprising the sorafenib analogue.

[00190] Figure 26 further demonstrates the acid responsiveness of a colloidal drug aggregate comprising the sorafenib analogue, with or without Herceptin as a stabilizing agent. In contrast, the colloidal drug aggregate comprising sorafenib, with or without Herceptin as a stabilizing agent, did not demonstrate acid responsiveness.

[00191] This study also included an investigation of the cytotoxicity of sorafenib in comparison to the cytotoxicity of the sorafenib analogue. As shown in Figure 27, the analogue exhibited similar (slightly increased) activity against all cancer cell lines tested.

[00192] Discussion

[00193] These experiments demonstrate that an aggregator (in this case a drug) that does not respond to acid can be made responsive by modification with an ionizable functional group, while retaining the pharmacologic activity of the aggregator.

[00194] EXAMPLE 4: Fulvestrant analogue-containing colloids

[00195] Methods

[00196] *Materials*

[00197] Fulvestrant was purchased from MedChemExpress. Dimethyl sulfoxide (DMSO), tert-butyldimethylsilyl chloride (TBSCl), (diacetoxyiodi)benzene, ammonium carbamate, N-Boc-glycine, N-Boc-valine, N,N-diisopropyl-N-ethylamine (DIPEA), tetra-N-butyl ammonium fluoride (TBAF) solution, tetrahydrofuran (THF), trifluoroacetic acid (TFA) bromoacetyl bromide, dimethylamine hydrochloride, and morpholine were purchased from Sigma-Aldrich. Dimethylformamide (DMF) was purchased from Alfa Aesar. Ethyl acetate, tert-butyl methyl ether (MTBE), hexanes, methanol, dichloromethane (DCM), ethanol, and glacial acetic acid were purchased from Caledon. Imidazole was purchased from Bio Basic. HCTU was purchased from AnaSpec. Silica (SilicaFlash P60™) was purchased from SiliCycle.

[00198] *Synthesis of ionizable fulvestrant analogues modified with tertiary amines*

[00199] Fulvestrant (species **1** in Figure 28 and Figure 29) analogues bearing tertiary amine functional groups were synthesized using a 5-step process summarized by Figure 28. Reagents and conditions: a) dihydropyran (3 equiv), TFA, DCM, rt 3 d; b) PhI(OAc)₂ (3 equiv), ammonium carbamate (4 equiv), methanol, rt, 4 h; c) bromoacetyl bromide, DCM, 0 °C → rt, 30 min; d) nitrogen nucleophile (NuH), or its hydrochloride salt (NuH₂HCl) and

DIPEA (5-20 equiv nucleophile), DCM, rt, 3-5 h; e) 2% TFA (>10 equiv) in methanol, rt, 16 h.

[00200] **3,17 β -Bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5,-pentafluoropentylsulphanyl) nonyl]estra-1,3,5-(10)-triene (2):** 3,4-Dihydro-2H-pyran (248 μ L, 2.72 mmol) and trifluoroacetic acid (12.6 μ L, 165 μ mol) were added under stirring to a solution of fulvestrant (500 mg, 824 μ mol) in dichloromethane (8 mL). The reaction was stirred at room temperature for 3 d, then extracted (1x sat. NaHCO₃, 1x brine), dried (MgSO₄), and filtered. The solvent was removed under reduced pressure, yielding a clear viscous oil which was used without further purification. R_f = 0.25 (1:1 hexanes:ethyl acetate).

[00201] **3,17 β -Bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5,-pentafluoropentyl sulphonimidoyl)nonyl] estra-1,3,5-(10)-triene (3):** Finely ground ammonium carbamate (85.2 mg, 1.09 mmol) and (diacetoxyiodo)benzene (221 mg, 685 μ mol) were added to a stirred solution of crude **2** (192 mg, $\leq$ 248 μ mol) in methanol (5 mL). After a few minutes, gas (presumably CO₂) was evolved and a yellow color developed. After 4 h, the reaction was diluted in ethyl acetate and extracted (2x PBS, 1x brine), dried (MgSO₄), and filtered. The solvent was removed and the product purified by column chromatography (silica, 1:1 hexanes:ethyl acetate) to give **3** as a clear oil (57.88 mg, 73.26 μ mol, 30% cumulative yield). R_f = 0.2 (1:1 hexanes:ethyl acetate); MS (ESI+) m/z [M+H]⁺ calculated for C₄₂H₆₅F₅NO₅S: 790.4511, found: 790.4503. Figure 30 shows the structure of **3** and its mass spectrum.

[00202] **N-(2-Bromoacetyl)-(3,17 β -bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5,-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene) (4):** **3** (57.9 mg, 73.3 μ mol) was dissolved in dichloromethane (2 mL) and N,N-diisopropylethylamine (63.8 μ L, 366 μ mol) added. The solution was cooled on ice, and then a solution of bromoacetyl bromide (12.8 μ L, 147 μ mol) in dichloromethane (2 mL) added under agitation. The now amber-coloured solution was allowed to warm to room temperature and stirred for 30 minutes. Then, 100 μ L of saturated Na₂CO₃ was added and the solvent removed under air. The residue was then dispersed in ethyl acetate and extracted (3x PBS, 1x brine), dried (MgSO₄), filtered, and evaporated under nitrogen. The crude product was purified by column chromatography (silica, 4:1 hexanes:ethyl acetate) to give **4** as a slightly yellow oil (40.5 mg,

44.1 μmol , 60%). $R_f = 0.4$ (4:1 hexanes:ethyl acetate), 0.85 (1:1 hexanes:ethyl acetate); ^1H NMR (500 MHz, CDCl_3) δ 7.17 – 7.10 (m, 1H), 6.63 (dd, $J = 8.4, 2.8$ Hz, 1H), 6.54 (d, $J = 3.1$ Hz, 1H), 5.05 (dt, $J = 5.2, 2.7$ Hz, 1H), 4.69 – 4.59 (m, 1H), 4.08 – 3.96 (m, 2H), 3.96 – 3.84 (m, 3H), 3.90 – 3.87 (m, 2H), 3.79 – 3.69 (m, 2H), 3.66 – 3.42 (m, 6H), 3.40 – 3.27 (m, 3H), 2.90 – 2.82 (m, 1H), 2.70 (dd, $J = 16.7, 4.8$ Hz, 1H), 2.38 – 1.08 (m, 27H), 1.08 – 0.85 (m, 6H), 0.83 – 0.76 (m, 4H); MS (ESI+) m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{44}\text{H}_{65}\text{BrF}_5\text{NNaO}_6\text{S}$: 932.3528, found: 932.3529. Figure 31 shows the structure of 4 and its mass spectrum.

[00203] **N-(2-Dimethylaminoacetyl)-(3,17 β -bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene) (5):** A solution of dimethylamine hydrochloride (285 mg) and DIPEA (70 μL , 400 μmol) in DCM (1.5 mL) was added to a solution of 4 (97 mg, 100 μmol) in 1 mL DCM. The reaction was agitated for 2.5 h, diluted with 10 mL ethyl acetate, and extracted (2x water, 1x brine). The extract was dried (MgSO_4) and the solvent removed under nitrogen. The resulting amber oil was used without further purification.

[00204] **N-(1H-Imidazol-1-ylacetyl)-(3,17 β -bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene) (6):** A solution of imidazole (16 mg, 0.23 mmol) in dichloromethane (1 mL) was added to 4 (18 mg, 20 μmol), agitated for 3 h, and then the solvent removed under nitrogen. The residue was then resuspended in ethyl acetate and extracted (2x water, 1x brine), dried (MgSO_4), and the solvent removed under nitrogen followed by vacuum. The resulting oil (18 mg, 20 μmol , 99%) was used without further purification. MS (ESI+) m/z $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{47}\text{H}_{69}\text{F}_5\text{N}_3\text{O}_6\text{S}$: 898.4822, found: 898.4820 ($[\text{M}+\text{H}-\text{THP}]^+$ and $[\text{M}+\text{H}-2\text{THP}]^+$ also observed). Figure 32 shows the structure of 6 and its mass spectrum.

[00205] **N-(Morpholin-4-ylacetyl)-(3,17 β -bis((tetrahydro-2H-pyran-2-yl)oxy)-7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene) (7):** A solution of morpholine (26 μL , 300 μmol) and DIPEA (17.42 μL , 100 μmol) in DCM (1.5 mL) was added to a solution of 4 (97 mg, 100 μmol) in 1 mL DCM. The reaction was agitated for 2.5 h, diluted with 10 mL ethyl acetate, and extracted (2x water, 1x brine). The extract was dried (MgSO_4) and the solvent removed under nitrogen. The resulting amber oil was used without further purification.

[00206] **N-(2-Dimethylaminoacetyl)-(7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene-3,17 β -diol) (8):** See method for **9**, but using **5** as in place of **6**. ^1H NMR (500 MHz, DMSO- d_6) δ 9.21 – 8.80 (s, 1H), 7.07 – 7.00 (d, 1H), 6.50 (dd, J = 8.4, 2.7 Hz, 1H), 6.41 (s, J = 2.6 Hz, 1H), 4.54 – 4.41 (m, 1H), 3.66 – 3.47 (m, 7H), 2.62 – 2.57 (m, 1H), 2.54 (s, 6H), 2.45 – 2.39 (m, 1H), 2.34 – 1.10 (m, 42H), 0.93 – 0.87 (m, 1H), 0.66 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 154.96, 135.97, 129.61, 126.63, 115.75, 112.85, 80.10, 61.72, 50.72, 49.40, 46.01, 43.81, 42.96, 41.74, 37.79, 36.78, 34.15, 32.76, 29.86, 29.37, 29.02, 28.72, 28.42, 27.81, 27.56, 27.41, 27.06, 25.81, 25.11, 22.28, 21.42, 13.55, 11.30; MS (ESI+) m/z $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{36}\text{H}_{56}\text{F}_5\text{N}_2\text{O}_4\text{S}$: 707.3875, found: 707.3876. Figure 33 and Figure 34 show, respectively, the ^1H and ^{13}C NMR spectra of **8**. Figure 35 shows the mass spectrum of **8**.

[00207] **N-(1H-Imidazol-1-ylacetyl)-(7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene-3,17 β -diol) (9):** A mixture of trifluoroacetic acid (20 μL) and methanol (480 μL) was added to a solution of crude **6** (13 mg, 15 μmol) in methanol (500 μL). The mixture was agitated overnight, diluted with ethyl acetate and extracted (1x sat. Na_2CO_3 , 1x brine), dried (MgSO_4), filtered, and evaporated under nitrogen. The crude product (7.9 mg) was then purified by column chromatography (silica, ethyl acetate followed by ethanol), dried under nitrogen, redissolved in acetonitrile, and filtered (0.45 μm PTFE) to yield an amber-tinted film (1.6 mg, 2.2 μmol , 15%) after drying under nitrogen. R_f = 0.1-0.3 (acetone), 0-0.2 (ethyl acetate), 0.9 (ethanol); ^1H NMR (500 MHz, DMSO- d_6) δ 8.97 (d, J = 6.6 Hz, 1H), 7.64 (s, 1H), 7.09 (s, 1H), 7.04 (d, J = 8.5 Hz, 1H), 6.89 (s, 1H), 6.50 (dd, J = 8.4, 2.6 Hz, 1H), 6.41 (d, J = 2.6 Hz, 1H), 4.77 (s, 2H), 4.48 (s, 1H), 3.65 – 3.43 (m, 6H), 2.46 – 0.78 (m, 33H), 0.66 (s, 3H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 174.99, 154.94, 135.99, 129.62, 126.64, 115.75, 112.85, 103.02, 80.09, 62.93, 51.18, 50.65, 49.31, 45.99, 42.96, 41.74, 37.78, 36.77, 34.15, 33.65, 32.75, 29.87, 29.36, 29.02, 28.72, 28.42, 27.79, 27.56, 27.41, 27.06, 25.10, 24.48, 22.28, 22.09, 21.43, 21.05, 18.39, 13.95, 13.53, 11.30; MS (ESI+) m/z $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{37}\text{H}_{53}\text{F}_5\text{N}_3\text{O}_4\text{S}$: 730.3671, found: 730.3676. Figure 36 and Figure 37 show, respectively, the ^1H and ^{13}C NMR spectra of **9**. Figure 38 shows the mass spectrum of **9**.

[00208] **N-(Morpholin-4-ylacetyl)-(7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene-3,17 β -diol) (10):** See

method for **9**, but using **7** in place of **6**. R_f = 0.1 (ethyl acetate), 0.8 (acetone), 0.9 (ethanol); ^1H NMR (500 MHz, DMSO- d_6) δ 8.97 (s, 1H), 7.04 (d, J = 8.5 Hz, 1H), 6.50 (dd, J = 8.4, 2.7 Hz, 1H), 6.41 (d, J = 2.6 Hz, 1H), 4.47 (d, J = 4.9 Hz, 1H), 3.66 – 3.40 (m, 10H), 3.04 (s, 2H), 2.60 (d, J = 16.6 Hz, 1H), 2.48 – 2.43 (m, 4H), 2.30 – 0.74 (m, 32H), 0.66 (s, 3H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 177.68, 154.94, 135.98, 129.62, 126.63, 115.75, 112.84, 80.09, 66.15, 63.27, 52.66, 50.57, 49.24, 45.99, 42.95, 41.74, 37.78, 36.76, 35.77, 34.14, 32.74, 31.29, 31.27, 30.76, 29.86, 29.59, 29.35, 29.00, 28.67, 28.39, 28.01, 27.84, 27.68, 27.54, 27.38, 27.06, 26.60, 25.10, 22.27, 22.09, 21.46, 13.95, 13.59, 11.30; MS (ESI+) m/z $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{38}\text{H}_{38}\text{F}_5\text{N}_2\text{O}_5\text{S}$: 749.3981, found: 749.3978. Figure 39 and Figure 40 show, respectively, the ^1H and ^{13}C NMR spectra of **10**. Figure 41 shows the mass spectrum of **10**.

[00209] *Synthesis of amino acid-modified fulvestrant*

[00210] Fulvestrant was modified by addition of a glycyl or valyl group at the sulfinyl to yield **15a** and **15b**, respectively, as shown in Figure 29. This modification was carried out using a 5-step procedure. First, fulvestrant (489 mg, 0.806 mmol), imidazole (538 mg, 7.90 mmol), and TBSCl (446 mg, 2.96 mmol) were dissolved in dry DMF and stirred at room temperature overnight. The solution was then diluted with MTBE and extracted with water and then saturated NaCl. The organic layer was dried over MgSO_4 and evaporated to yield species **11** in Figure 29. Second, the TBS-protected fulvestrant was dissolved in methanol, then solid ammonium carbamate (236 mg, 3.22 mmol) and (diacetoxyiodo)benzene (712 mg, 2.42 mmol) were added under stirring. The mixture was stirred at room temperature for 4 h, then diluted with ethyl acetate and extracted with water followed by saturated NaCl. The organic layer was dried over MgSO_4 and evaporated to yield species **12** in Figure 29. This material was purified by column chromatography using 1:1 hexanes:ethyl acetate on silica (R_f = 0.4) to yield 547 mg of sticky oil (80% yield from **1**). Third, N-Boc amino acid (60 mmol) and HCTU (25 mg, 60 mmol) were dissolved in DMF and cooled on ice. DIPEA (23 mg, 180 mmol) was added and the mixture was stirred at room temperature for 15 min. The mixture was then added to **6** (42 mg, 50 mmol) and vortexed to dissolve it, then stirred overnight. The reaction mixture was then diluted with MTBE and extracted with lightly salted water (5x) and saturated NaCl (1x). The organic layer was dried over MgSO_4 and evaporated to yield **13** as a sticky yellow oil. This material was purified by column chromatography using 3:1

hexanes:ethyl acetate on silica ($R_f = 0.4$). Fourth, **13** (1 eq.) was dissolved in THF and acetic acid (5 eq.) and TBAF (5 eq.) as a 1 M solution in THF were added. This mixture was heated at 60 °C for 4 h then evaporated. The resulting resin was dissolved in ethyl acetate and extracted with 0.1 M NaHCO_3 followed by saturated NaCl. The organic layer was evaporated, then purified by column chromatography using 2:1 hexanes:ethyl acetate to yield **14**. Fifth, a solution containing 78% (v/v) DCM, 20% (v/v) TFA, and 2% (v/v) water was prepared and chilled on ice. This solution was added to **14** and stirred on ice for 15 min, then at room temperature for 2 h. The reaction mixture was then added dropwise to saturated, ice cold Na_2CO_3 and stirred for 15 min before diluting with water and ethyl acetate. The organic layer was isolated, dried over MgSO_4 , and evaporated to yield crude **2**. The product was then dissolved in acetonitrile and purified by HPLC using a 50:50 water:acetonitrile with 0.1% TFA to 100% acetonitrile with 0.1% TFA gradient on a C18-modified silica column. The resulting TFA salt of **15** was dissolved in MTBE and extracted with saturated Na_2CO_3 . The organic layer was isolated, dried over MgSO_4 , and evaporated to yield pure **15**. Figure 42 shows the ^1H spectrum of **15a**; Figures 43 and 44 provide the mass spectra for **15a** and **15b** confirming successful synthesis of these analogues.

[00211] **N-(2-aminoacetyl)-(7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonimidoyl)nonyl]estra-1,3,5-(10)-triene-3,17 β -diol) (9):** ^1H NMR (500 MHz, DMSO-d_6) δ 7.04 (d, $J = 8.5$ Hz, 1H), 6.50 (dd, $J = 8.5, 2.6$ Hz, 1H), 6.41 (d, $J = 2.6$ Hz, 1H), 4.48 (s, 1H), 3.66 – 3.42 (m, 5H), 3.23 (s, 2H), 2.75 (dd, $J = 16.6, 5.2$ Hz, 1H), 2.63 – 2.55 (m, 1H), 2.47 – 2.34 (m, 2H), 2.25 (dd, $J = 12.1, 4.2$ Hz, 1H), 2.21 – 2.11 (m, 1H), 2.05 – 1.58 (m, 7H), 1.58 – 1.13 (m, 22H), 0.97 – 0.79 (m, 1H), 0.66 (s, 3H); MS (ESI+) m/z $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{34}\text{H}_{52}\text{F}_5\text{N}_2\text{O}_4\text{S}$: 679.3562, found: 679.3559.

[00212] *Colloid formulation and determination of CAC*

[00213] Colloids were formulated as described in Example 1. Briefly, solutions of fulvestrant analogue in DMSO were prepared at various concentrations and then diluted 100-fold with PBS at different concentrations. 100 μL of each sample was analyzed by DLS and the CAC's calculated as described in Example 1.

[00214] Results

[00215] Figure 45 shows a comparison of the CACs for fulvestrant and acid-responsive analogues of fulvestrant over a range of pH values. The increase in CAC values found for the fulvestrant analogues as the pH decreases is indicative of the acid responsiveness of the colloidal drug aggregates comprising the fulvestrant analogues. Importantly, the acid-responsive fulvestrant analogues were more soluble at lysosomal pH (4) than at extracellular pH (7.4). No change in CAC values were observed for fulvestrant with changing pH.

[00216] Discussion

[00217] As in Example 3, this study demonstrates that an aggregator that does not respond to acid can be made acid-responsive by modification with an ionizable functional group. Furthermore, these results show that the pKa of the aggregator, and thus the pH at which it becomes ionized, can be tuned by adjusting the nature of the attached ionizable functional group.

[00218] EXAMPLE 5: Siramesine colloids

[00219] Methods

[00220] *Materials*

[00221] Siramesine hydrochloride was purchased from MedChemExpress. Bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) was purchased from Wisent. Hydrochloric acid was purchased from VWR.

[00222] *Colloid formulation and CAC measurement*

[00223] Colloids were formulated as described in Example 1. Briefly, solutions of siramesine in DMSO were prepared at various concentrations and then diluted 100-fold with pH-adjusted PBS containing stabilizer (where applicable). 100 µL of each sample was analyzed by DLS and the CACs calculated as described in Example 1.

[00224] *High concentration colloid formulation*

[00225] Siramesine was dissolved in DMSO at a concentration of 50 mg/mL, or in ethanol at a concentration of 10 mg/mL. 15 μ L (DMSO) or 75 μ L (ethanol) of this solution was diluted with 1.5 mL of water containing 5 mg/mL of BSA. The resulting colloids were flash frozen and lyophilized to yield a white cake. The lyophilized colloids were reconstituted by adding 150 μ L of PBS and mixing with a pipette. The reconstituted colloids were measuring by DLS after diluting 10 μ L with 90 μ L of PBS.

[00226] Results

[00227] Figure 46 shows a comparison of the CACs for siramesine over a range of pH values. The increase in CAC values found for siramesine as the pH decreases is indicative of the acid responsiveness of the colloidal drug aggregate comprising the siramesine.

[00228] Figure 47 and Figure 48 demonstrate stabilization of siramesine colloids using polysorbate 80 or bovine serum albumin excipients.

[00229] Discussion

[00230] These results demonstrated that the colloid-forming compound siramesine is acid-responsive, and that colloids of siramesine can be formulated at high concentrations.

[00231] EXAMPLE 6: Diphtheria toxin-stabilized sorafenib colloids

[00232] Methods

[00233] *Materials*

[00234] Sorafenib was purchased from MedChemExpress. Bovine serum albumin (BSA) and RPMI 1640 cell culture media were purchased from Sigma-Aldrich. Attenuated diphtheria toxin (aDT) was obtained from Professor Roman Melnyk's (The Hospital for Sick Children, Toronto, ON, Canada). SKOV3 cells were purchased from ATCC. Fetal bovine serum was purchase from Wisent Bio Products. PrestoBlue™ cell viability reagent was purchased from Thermo Fisher Scientific.

[00235] *Colloid formulation and characterization by dynamic light scattering*

[00236] Colloidal drug aggregates were formulated as described as described in Example 1 using sorafenib in place of lapatinib. In particular, colloids of sorafenib were formulated at 50 μM or 200 μM and stabilized with 10 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$ diphtheria toxin in phosphate buffered saline. These formulations were assessed by dynamic light scattering as described in Example 1.

[00237] *Cell culture*

[00238] SK-OV-3 cells were maintained in a humidified incubator at 37°C with 5% atmospheric CO_2 . Cells were grown in 75 cm^2 tissue culture flasks with 10 mL RPMI 1640 supplemented with 10% FBS, 10 UI/mL penicillin, and 10 $\mu\text{g mL}^{-1}$ streptomycin. Cells were passaged twice per week with typical subculture ratio of 1:5.

[00239] *Cell viability experiments*

[00240] After passaging, cell suspensions were diluted into fresh media and 100 μL was pipetted into each well of a 96 well plate. Five thousand cells per well were plated and allowed to adhere overnight. Then, the media was withdrawn and replaced with treatment formulations (prepared as described above) or control treatments. Cells were treated with either cell culture medium, bovine serum albumin (BSA; 100 $\mu\text{g/mL}$), aDT (100 $\mu\text{g/mL}$), BSA-stabilized sorafenib colloids (100 $\mu\text{g/mL}$ BSA; 200 μM sorafenib) or aDT-stabilized sorafenib colloids (100 $\mu\text{g/mL}$ aDT; 200 μM sorafenib). All treatments were prepared in complete culture media. Cells were incubated during the experiment in a humidified incubator at 37°C with 5% atmospheric CO_2 . For treatments lasting less than 3 d, the treatment solutions were removed after the prescribed time, cells were washed with fresh media, and 200 μL of fresh media was added. Cell viability was assessed 3 days after commencing treatment using the PrestoBlue™ viability assay according to the manufacturer's protocol. Cell viability was reported as a percentage of the blank (cell culture medium) control.

[00241] Results

[00242] Figure 49 shows a time course of hydrodynamic diameter of sorafenib colloids formulated with varying concentrations of aDT as a stabilizing excipient. These colloids are stable for at least 48 hours in phosphate buffered saline at 37°C (simulating physiological conditions).

[00243] Figure 50 demonstrates cytotoxicity of sorafenib colloids (200 µM) coated with attenuated diphtheria toxin (100 µg/mL) to an ovarian cancer cell line (SK-OV-3). aDT-stabilized sorafenib colloids (Sor-DT) reduced cell metabolic activity compared to control BSA-stabilized sorafenib colloids (Sor-BSA). Treatment with either BSA or aDT alone did not cause a reduction in cell viability.

[00244] Discussion

[00245] These results demonstrated that colloidal drug aggregates can be stabilized by an acid-responsive protein stabilizer/excipient (e.g., aDT), and this coating enhances cytotoxicity against a cancer cell line.

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[00247] All publications, patents and patent applications mentioned in this Specification are indicative of the level of skill of those skilled in the art to which this invention pertains and are herein incorporated by reference to the same extent as if each individual publication, patent, or patent applications was specifically and individually indicated to be incorporated by reference.

[00248] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

WE CLAIM:

1. An acid-responsive composition comprising: a colloidal aggregate of one or more drugs and a stabilizing agent, wherein the colloidal aggregate disrupts, dissolves or disassembles when the acid-responsive composition is in an acid environment having a pH of less than 7.4.
2. The acid responsive composition according to claim 1, wherein the acid environment has a pH of less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4.
3. The acid responsive composition according to claim 1 or 2, wherein:
 - (a) at least one of the one or more drugs is an ionizable drug or ionizable drug analogue, wherein the conjugate acid of the ionizable drug or drug analogue has a pKa of at least 4, or at least 4.5 or at least 5, or at least 5.5, or at least 6, or at least 6.5; and/or
 - (b) the stabilizing agent is an acid-responsive stabilizing agent such that the stabilizing agent undergoes a morphological and/or functional change when pH is reduced to less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4.
4. The composition according to any one of claims 1 to 3, wherein at least one of the one or more drugs is an ionizable drug or ionizable drug analogue, wherein the conjugate acid of the ionizable drug or drug analogue has a pKa of at least 4, or at least 4.5 or at least 5, or at least 5.5, or at least 6, or at least 6.5.
5. The composition according to claim 4, wherein the stabilizing agent is morphologically and/or functionally stable in acid conditions.
6. The composition according to any one of claims 1 to 4, wherein the stabilizing agent is an acid-responsive stabilizing agent such that the stabilizing agent undergoes a morphological and/or functional change when pH is reduced to less than about 6.5, or less than about 6, or less than about 5.5, or less than about 5, or less than about 4.5, or less than about 4.

7. The composition according to any one of claims 1 to 6, wherein the stabilizing agent is a protein (e.g. an antibody, or antibody fragment, or an attenuated diphtheria toxin), a polymer, a colloid-forming compound (e.g., vitamin E) or another colloid-forming drug (e.g., fulvestrant).
8. The composition according to claim 7, wherein the stabilizing agent is an attenuated diphtheria toxin or a derivative thereof.
9. The composition according to claim 7, wherein said other colloid-forming drug is not ionizable at a pH of 4 or less.
10. The composition according to claim 7, wherein the protein is IgG, trastuzumab, albumin, or transferrin.
11. The composition according to claim 7, wherein the polymer is a polymeric surfactant, such as UP80, PLAC-PEG, Brij 58, F127, Vitamin E-PEG, F68, or Brij L23.
12. The composition according to any one of claims 1 to 11, wherein said one or more drugs comprises lapatinib, clotrimazole, nilotinib, pazopanib, or siramesine.
13. The composition according to any one of claims 1 to 12, wherein when the colloidal aggregate disrupts, dissolves or disassembles when the acid-responsive composition is in the acid environment the one or more drugs are released.
14. The composition according to any one of claims 1 to 13, wherein the acidic environment is stomach acid, or a lysosome or an endosome of a cell.
15. The composition according to any one of claims 1 to 14, which further comprises a targeting compound for delivery of the composition to a target site.
16. The composition according to claim 15, wherein the targeting compound is a binding protein, a specific antibody or antibody fragment, or a binding molecule, wherein the targeting compound selectively binds to a cell receptor.
17. The composition according to claim 16, wherein the targeting compound is transferrin, trastuzumab, diphtheria toxin, attenuated diphtheria toxin, or a variant thereof.

18. The composition according to any one of claims 15 to 17, wherein the the targeting compound is functions together with the stabilizing agent to stabilize the colloidal drug aggregate.
19. The composition according to claim 3, wherein the ionizable drug is lapatinib, the stabilizing agent is fulvestrant, and the composition further comprises transferrin as a targeting compound.
20. The composition according to claim 3, wherein the ionizable drug is lapatinib and the stabilizing agent is a combination of fulvestrant and a polymeric surfactant.
21. The composition according to claim 20, wherein the polymeric surfactant is PLAC-PEG.
22. The composition according to any one of claims 1 to 11, wherein said one or more drugs comprises an ionizable drug analogue, which comprises a drug molecule chemically modified to include an ionizable moiety.
23. The composition according to claim 22, wherein the drug molecule is sorafenib or fulvestrant.
24. The composition according to claim 22 or 23, wherein the ionizable drug analogue is pharmaceutically active.
25. The composition according to claim 22 or 23, wherein the ionizable drug analogue is a prodrug and the ionizable drug moiety is cleavable following ionization.
26. The composition according to claim 3, wherein the drug is sorafenib and the acid-responsive stabilizer is attenuated diphtheria toxin.
27. The composition according to any one of claims 1 to 26 for use in a drug therapy.
28. A method for drug delivery to a target site in a subject, comprising administering to the subject an acid-responsive composition according to any one of claims 1 to 26, wherein disruption, dissolution or disassembly of the colloidal aggregate results in delivery of at least one of the one or more drugs to the target site in the subject.

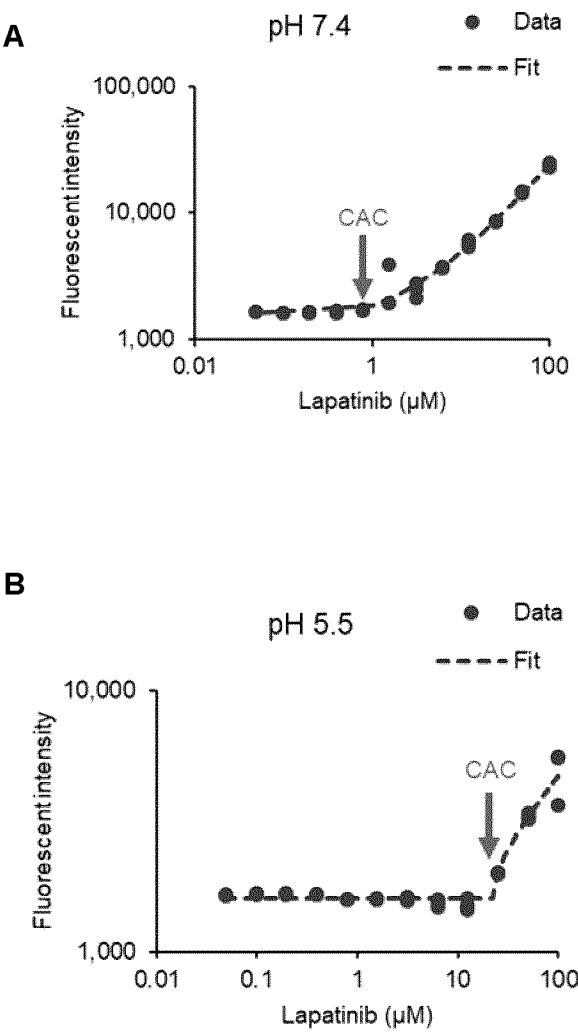


Figure 1

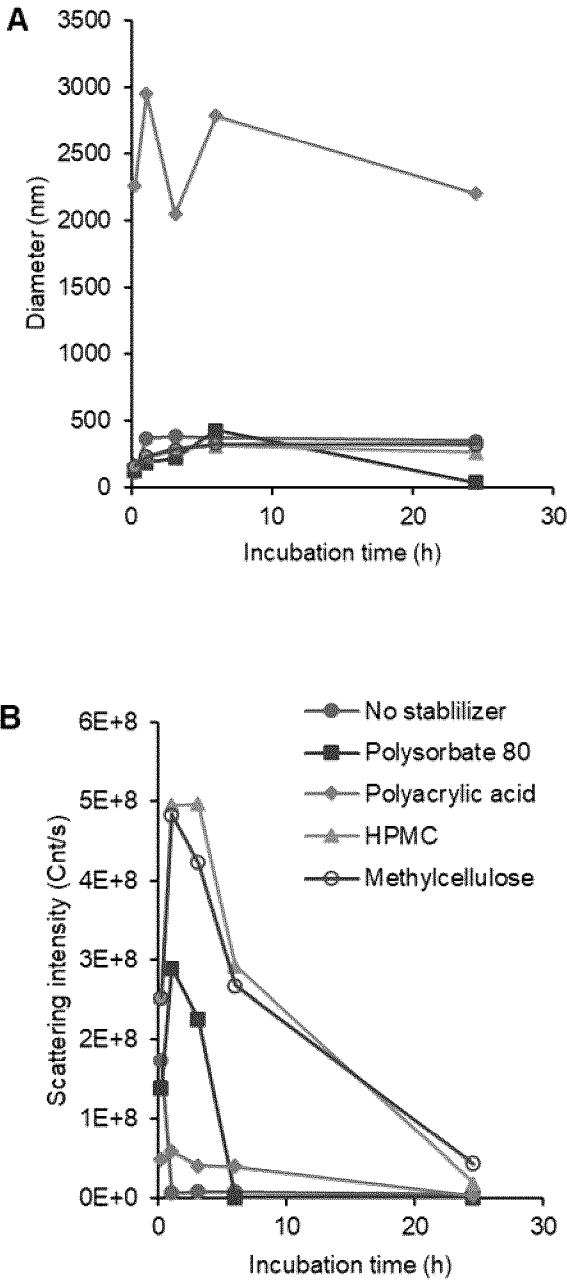


Figure 2

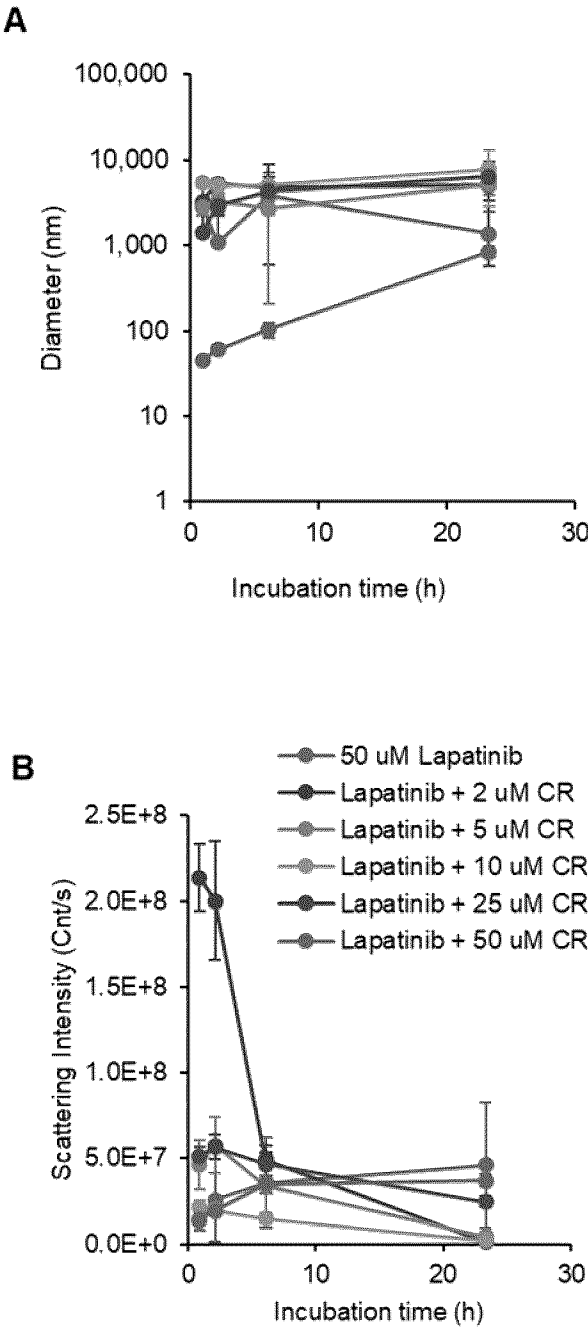


Figure 3

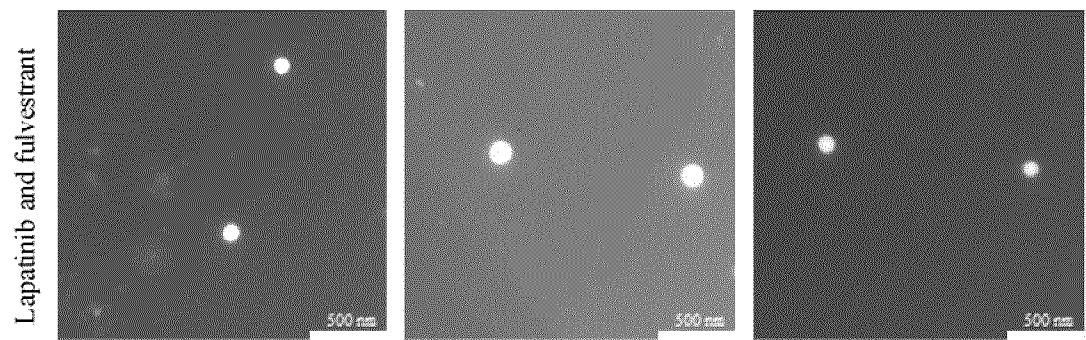


Figure 4

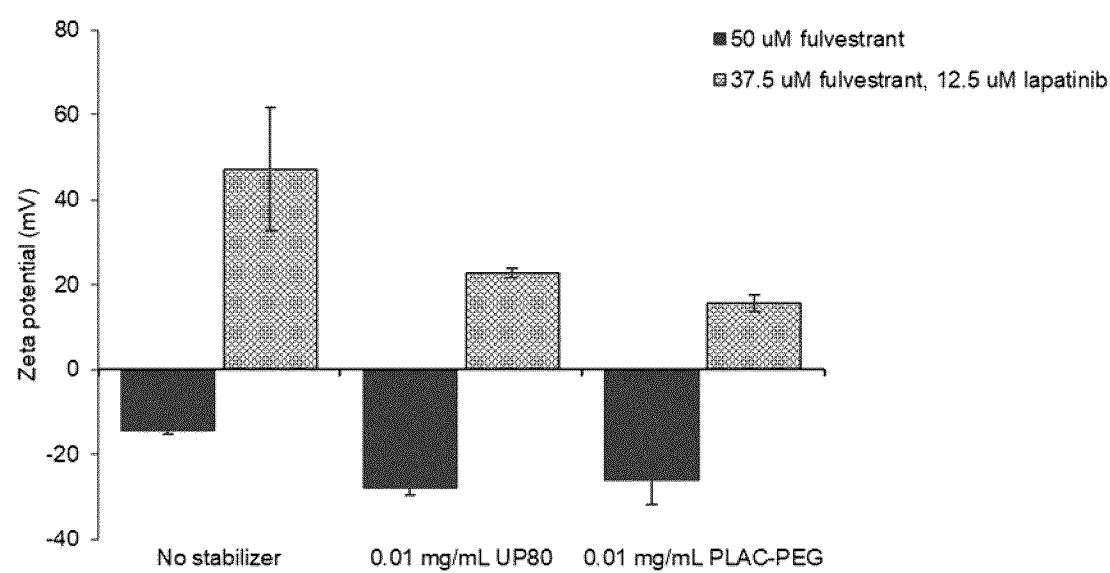


Figure 5

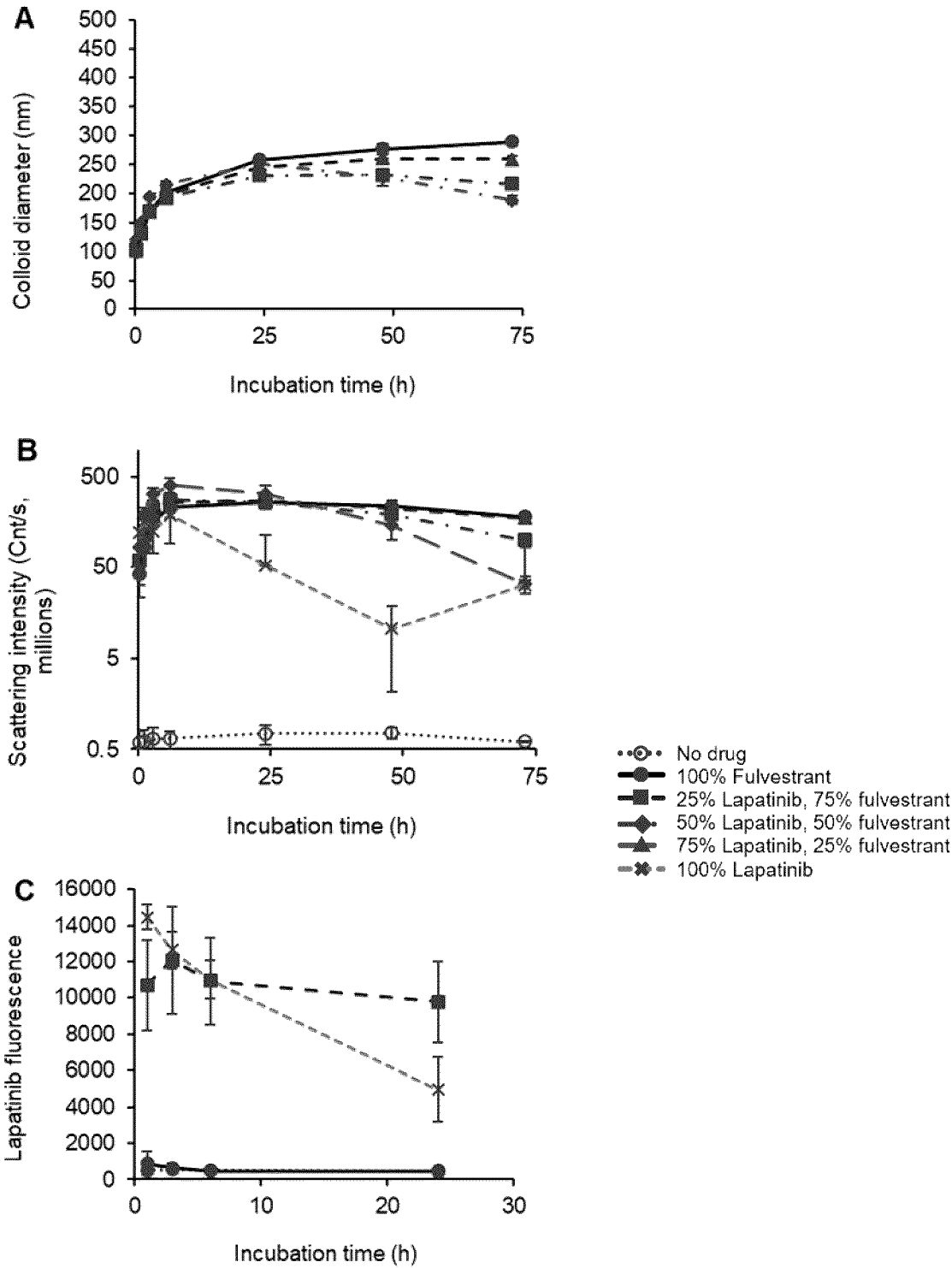


Figure 6

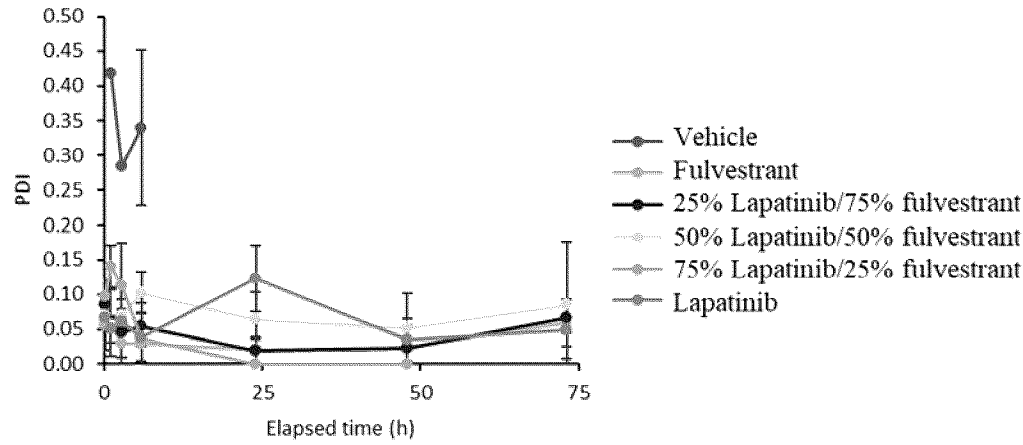


Figure 7

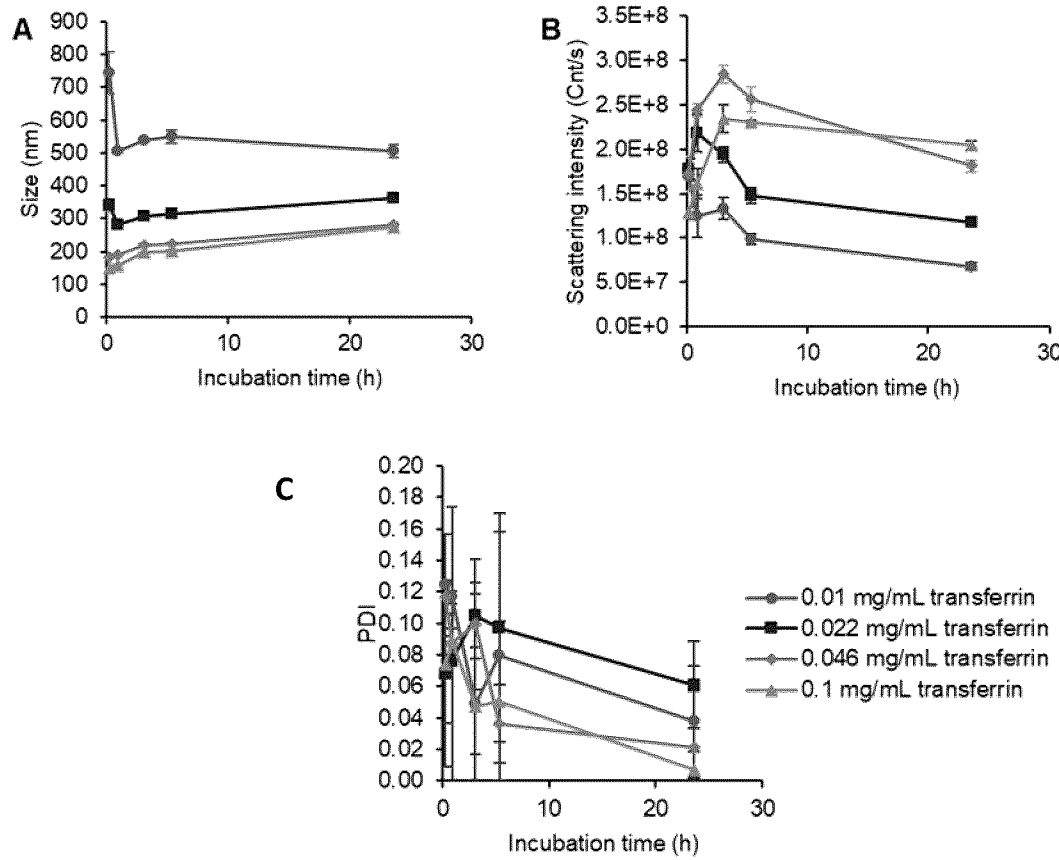


Figure 8

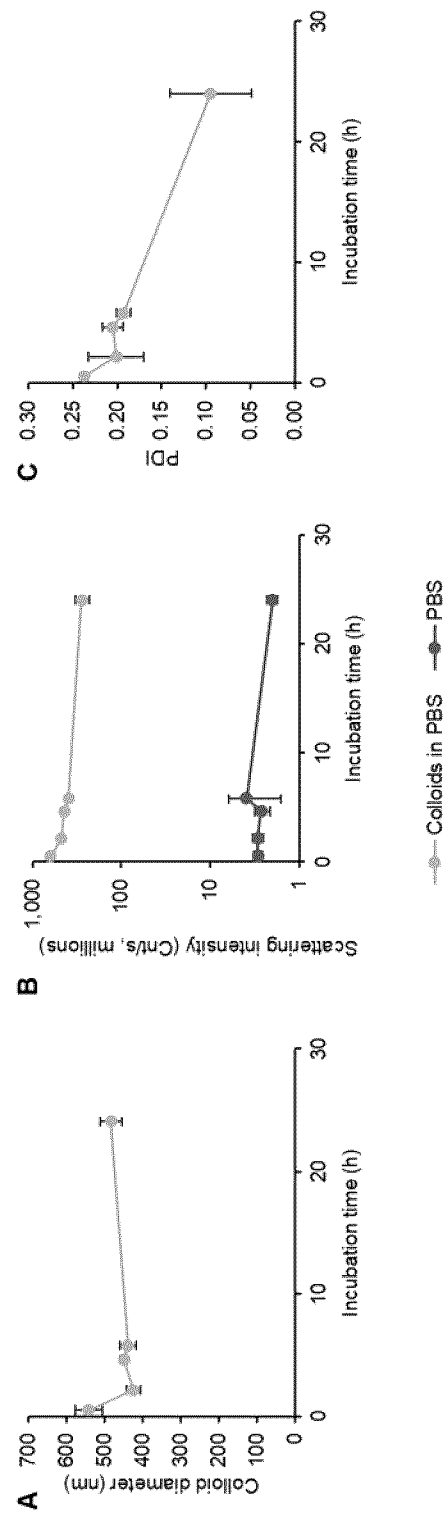


Figure 9

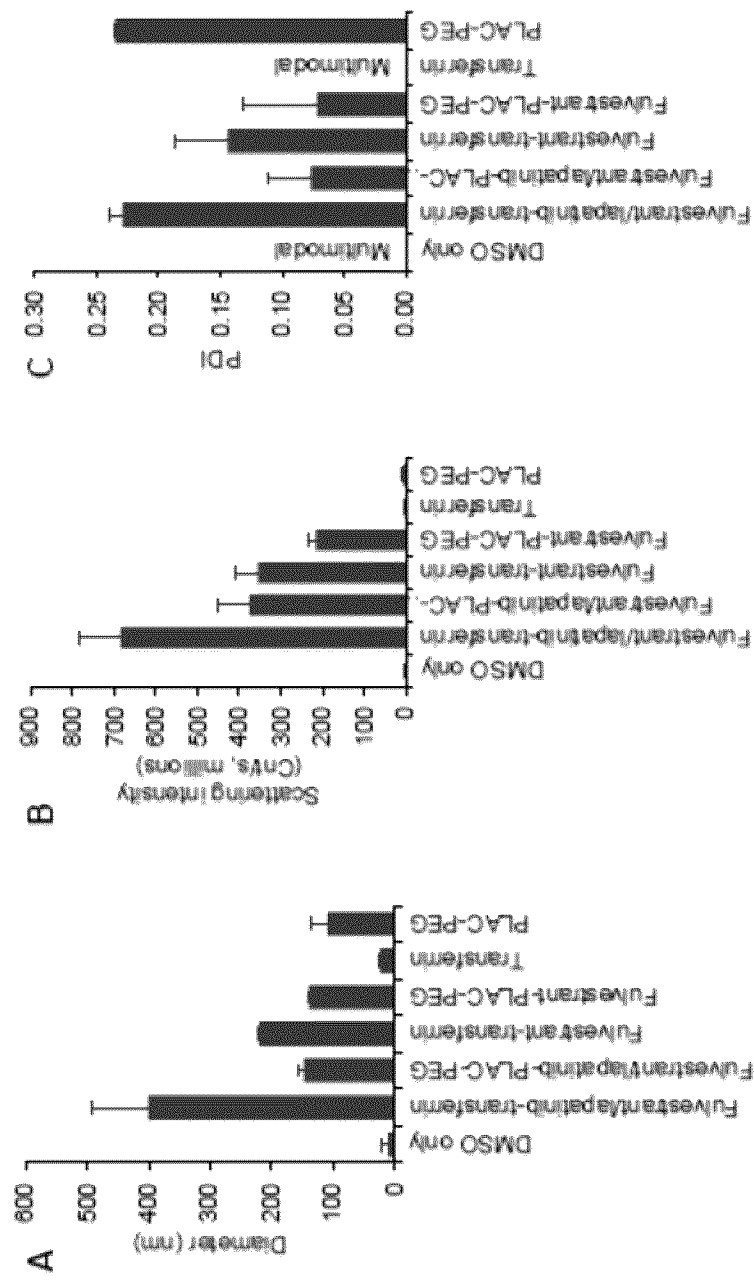


Figure 10

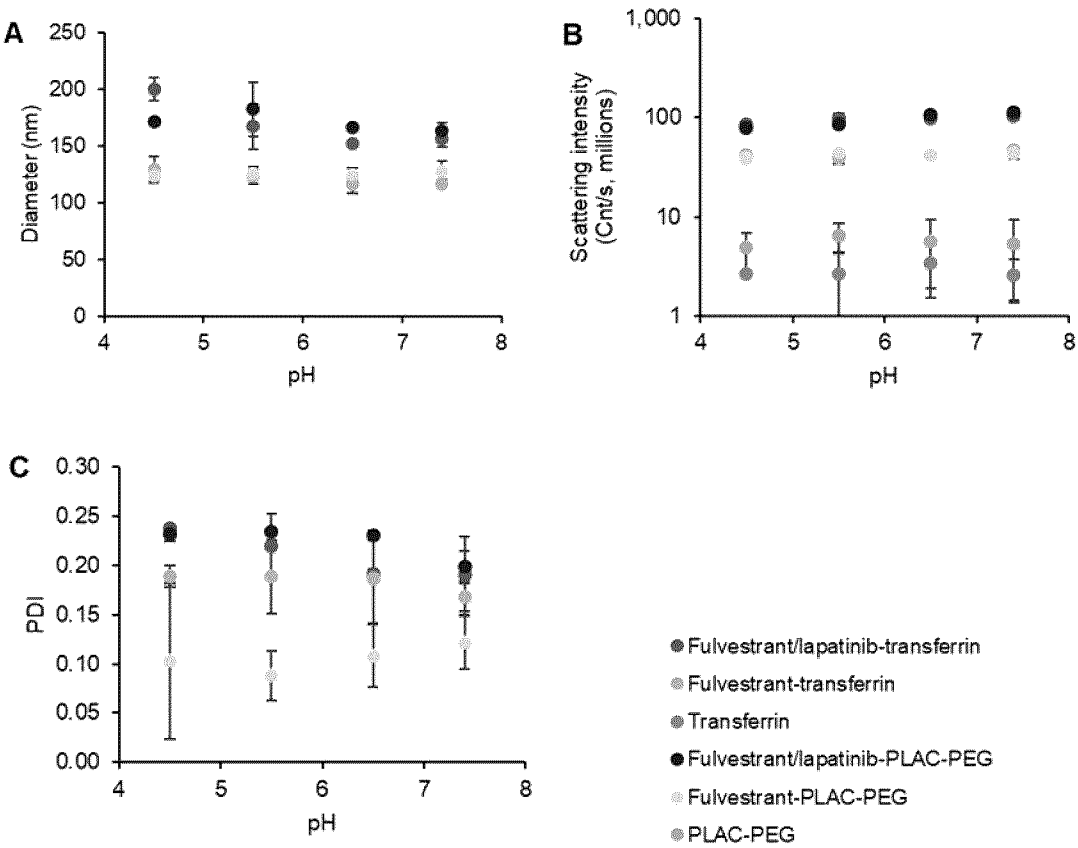


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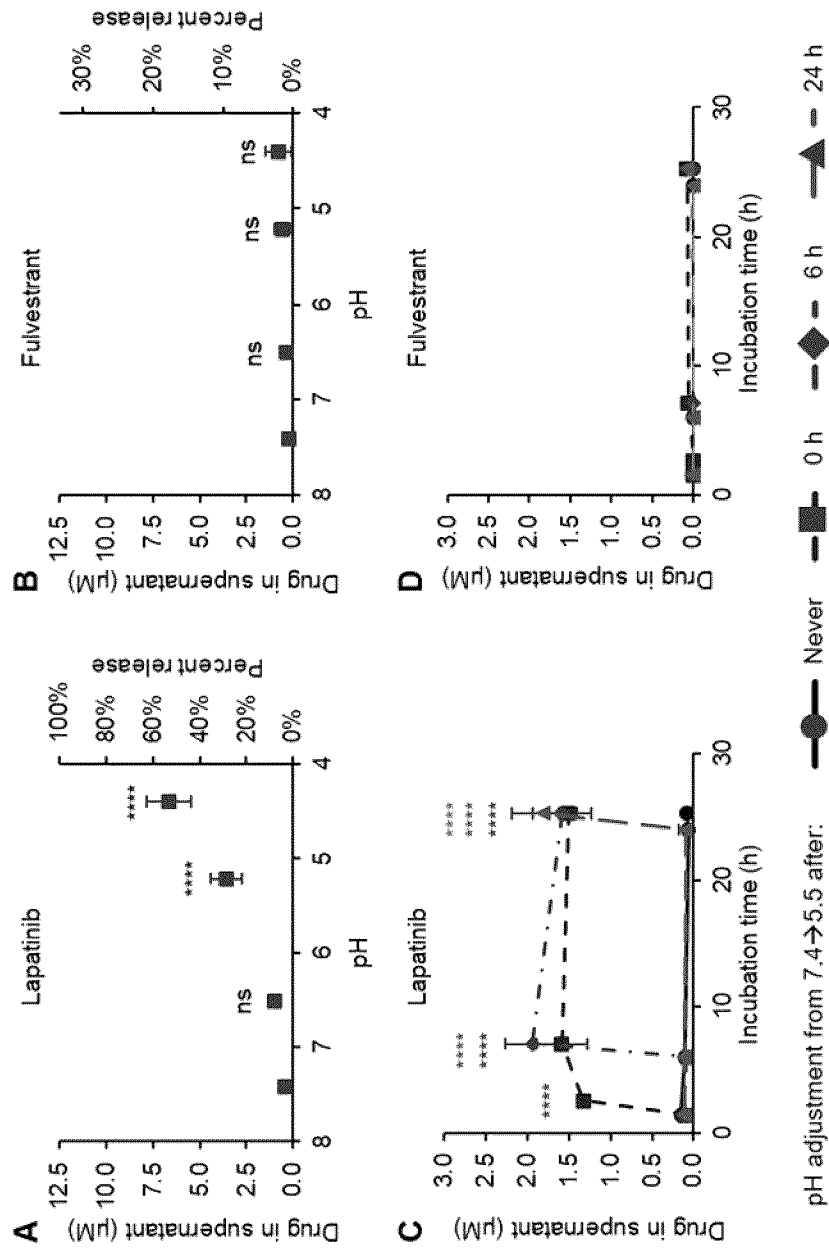


Figure 12

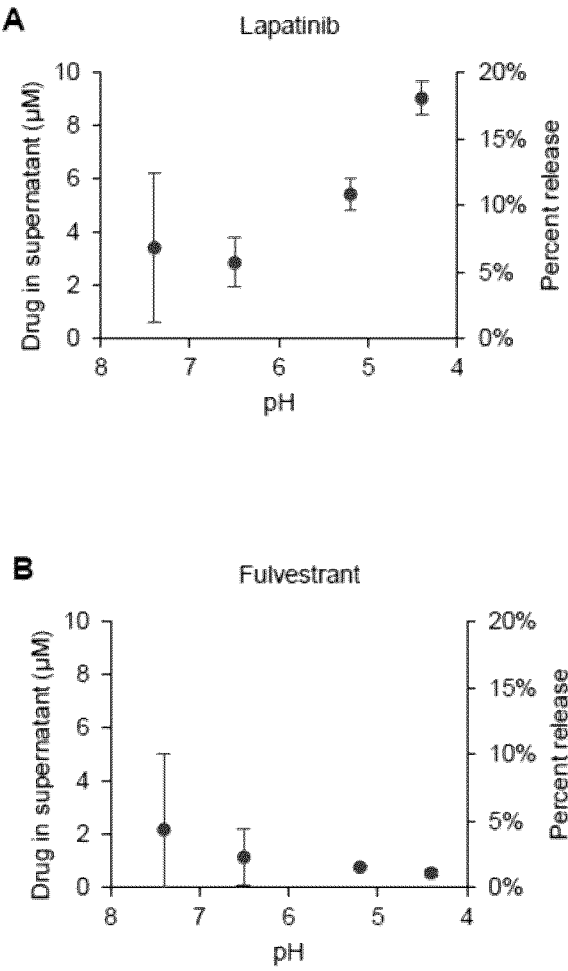


Figure 13

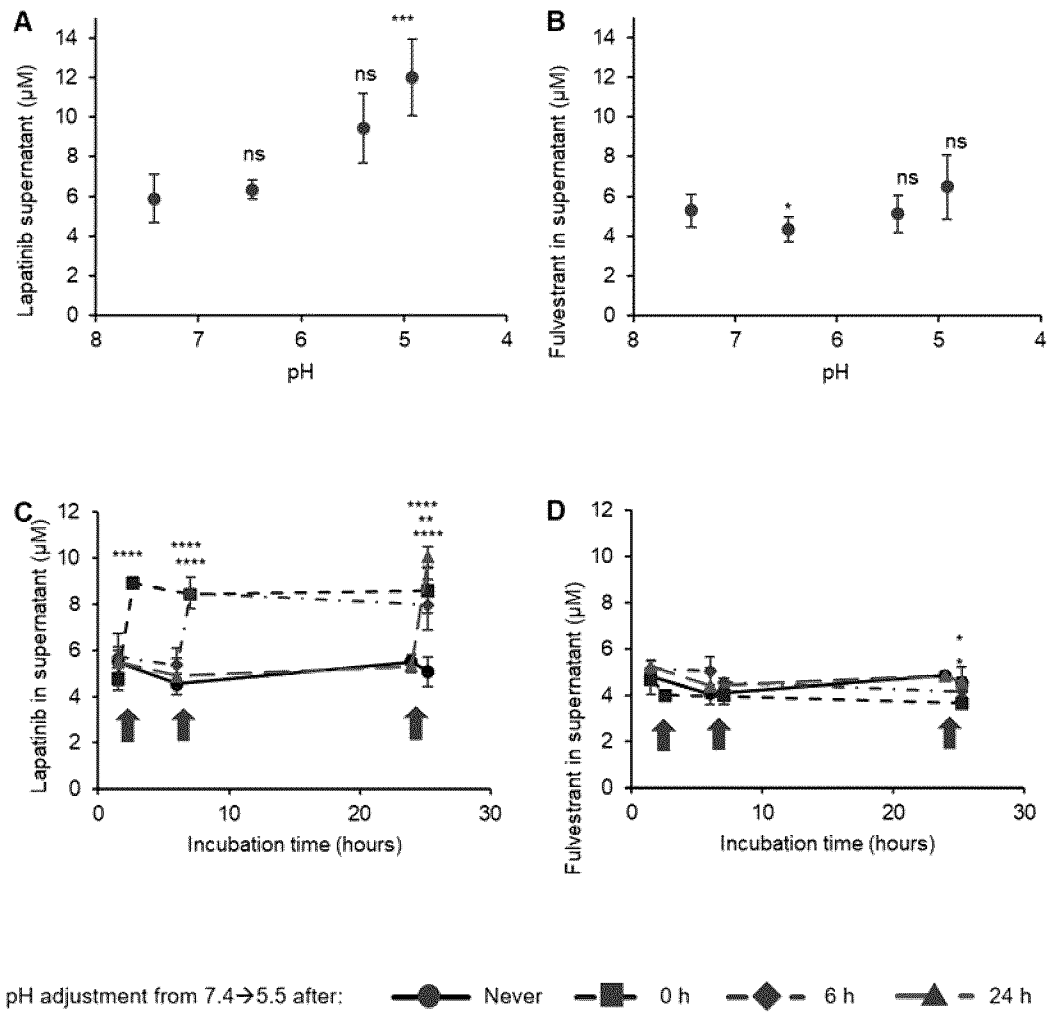


Figure 14

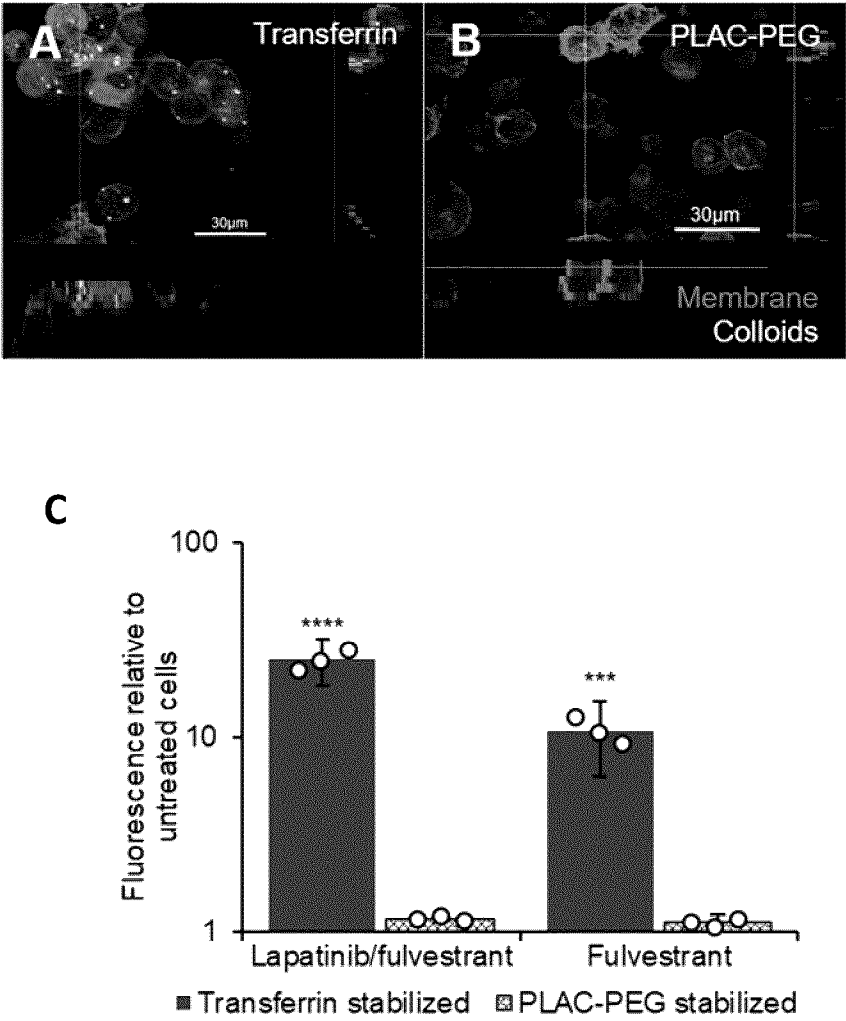


Figure 15

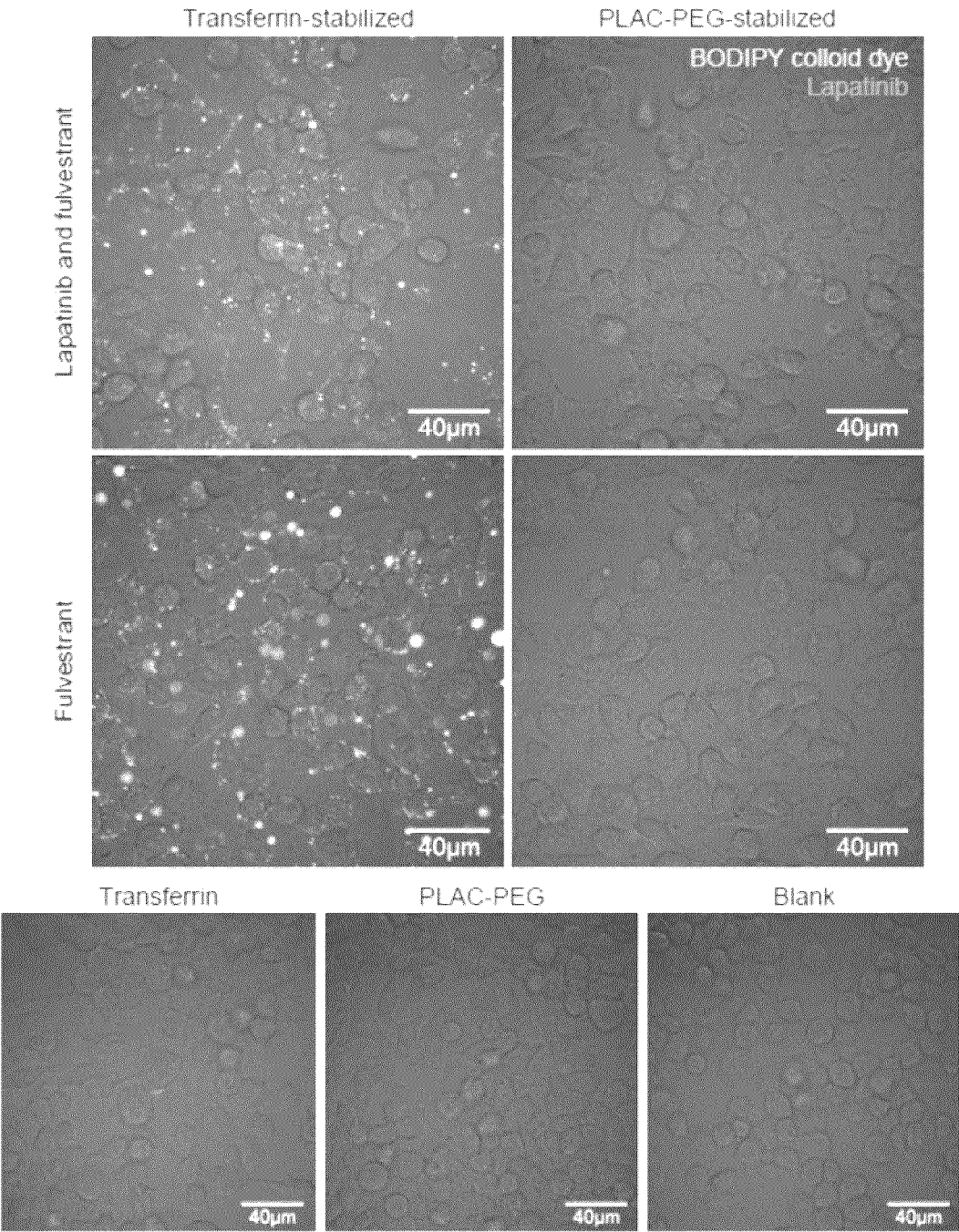


Figure 16

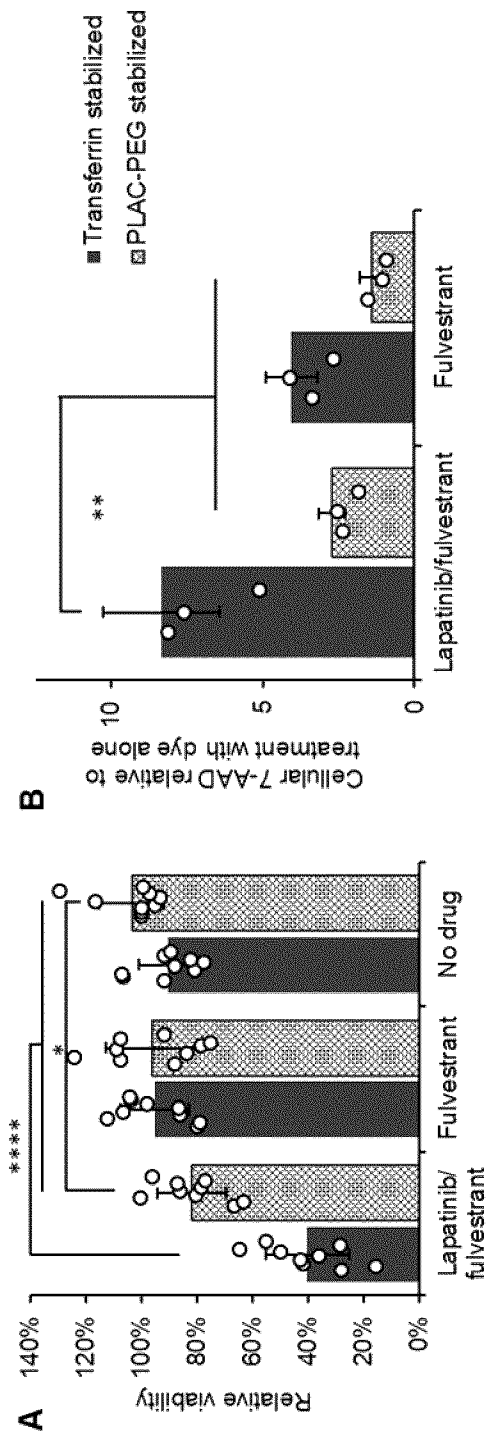


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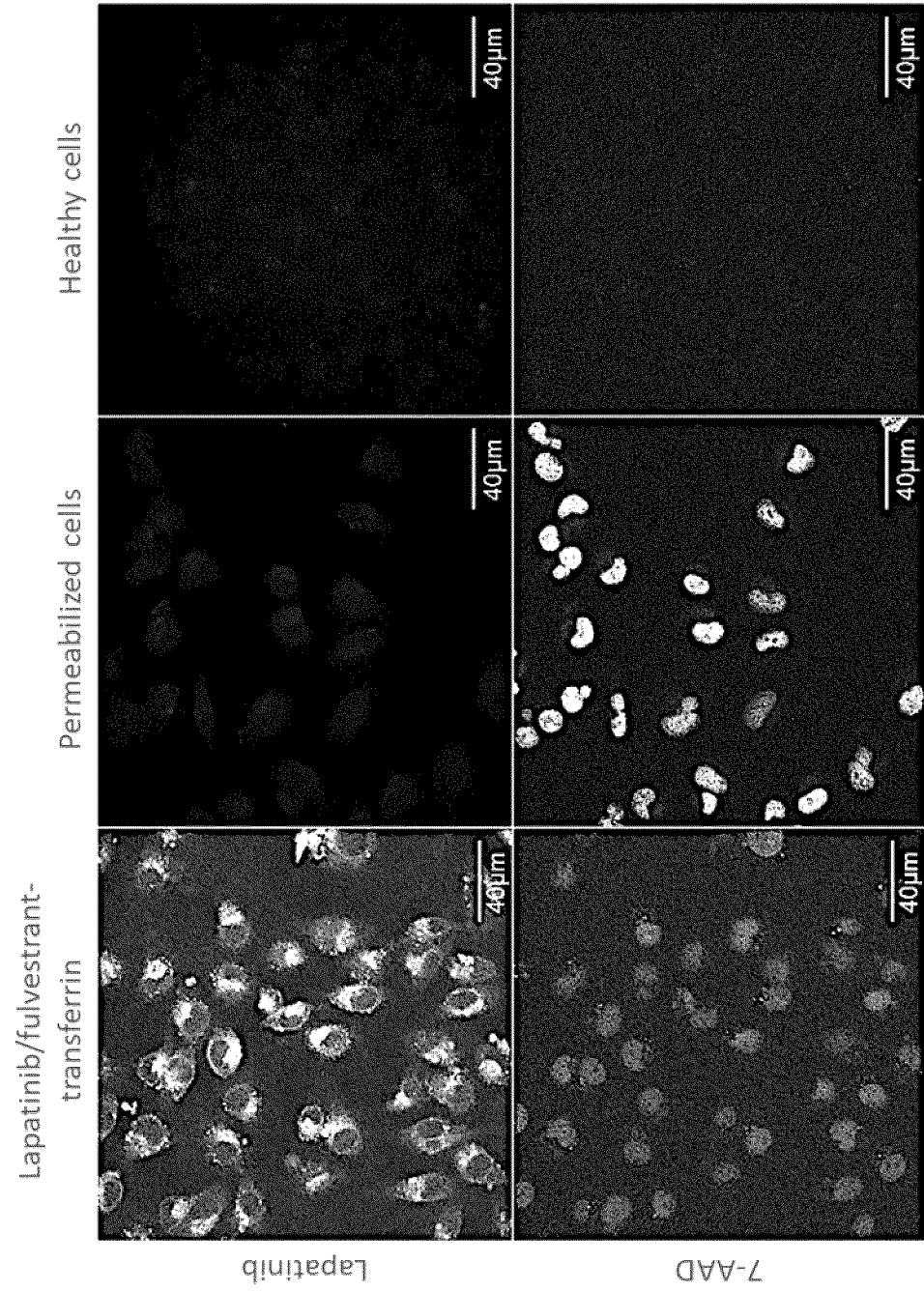


Figure 17C

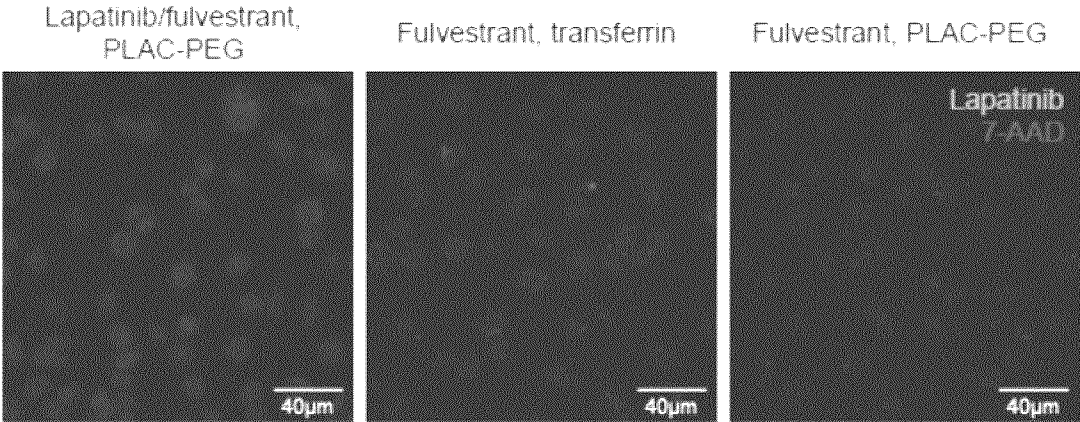


Figure 18

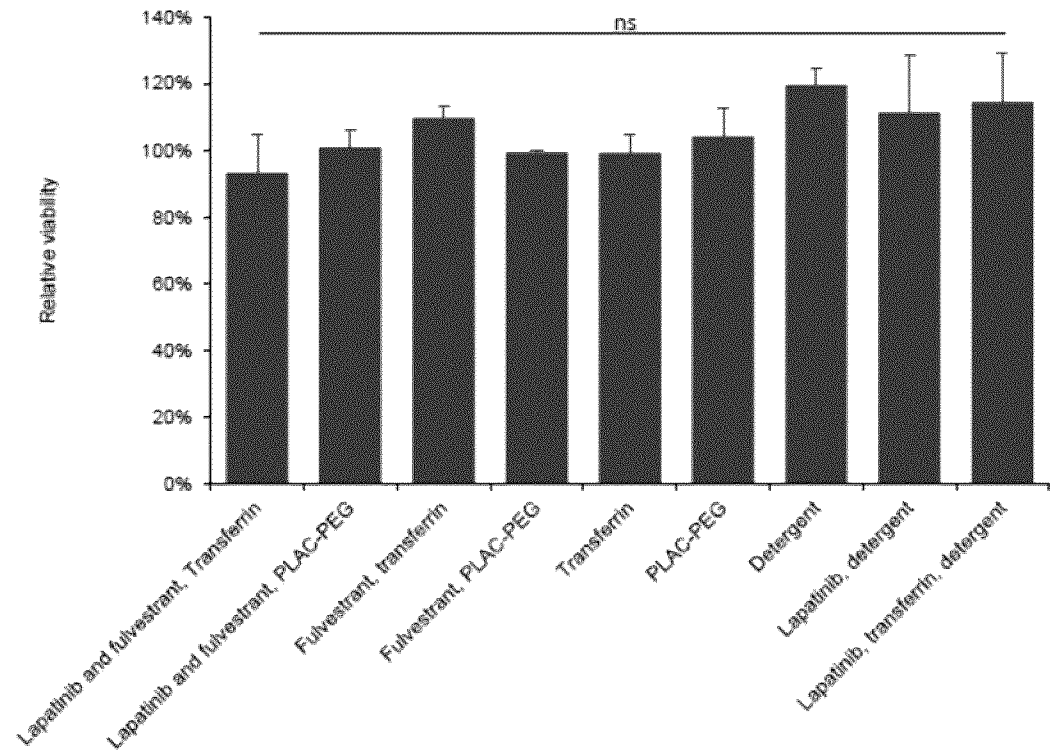


Figure 19

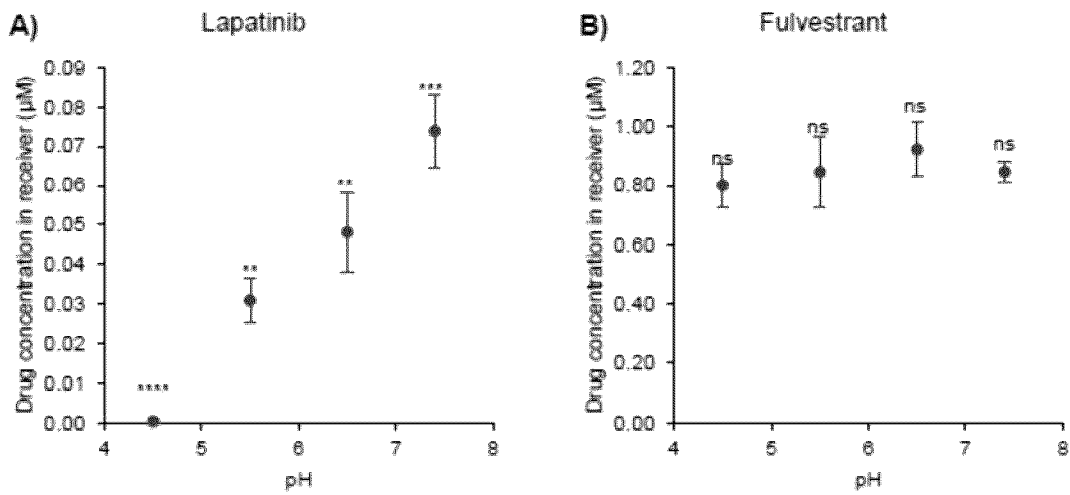


Figure 20

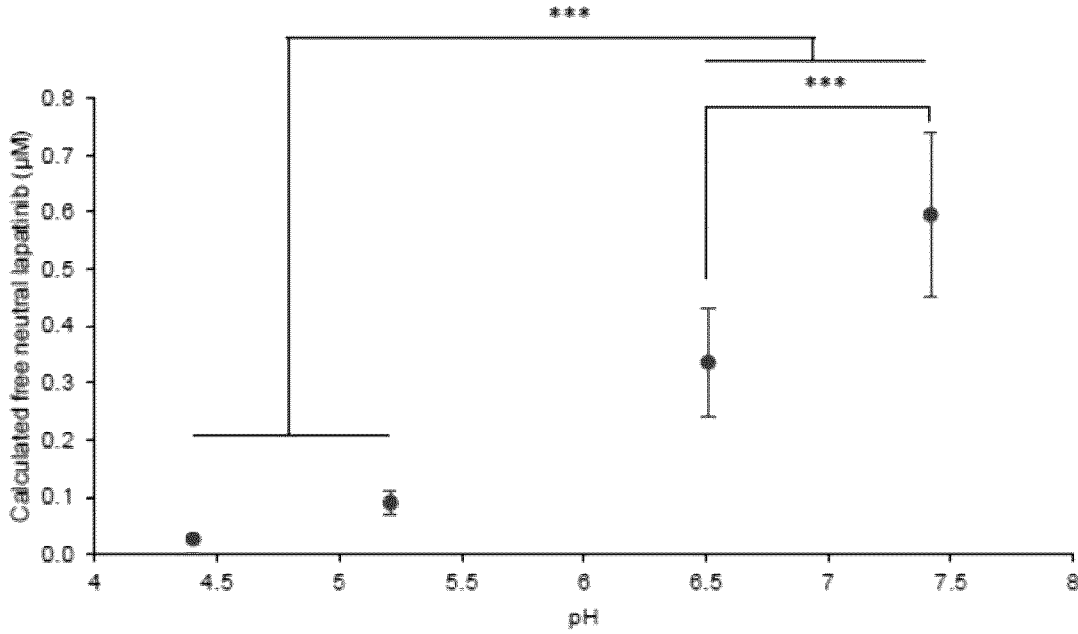


Figure 21

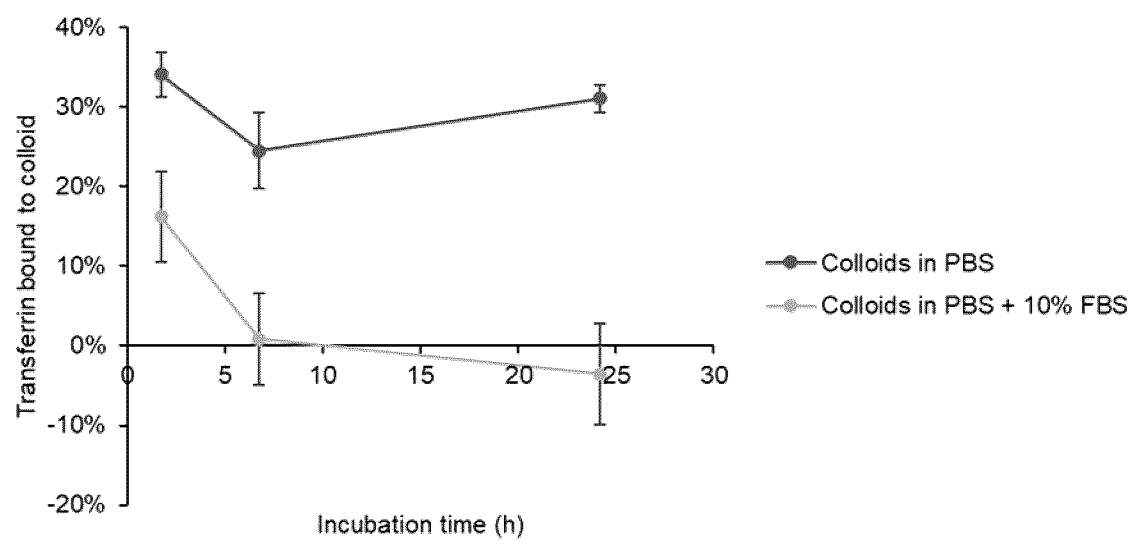


Figure 22

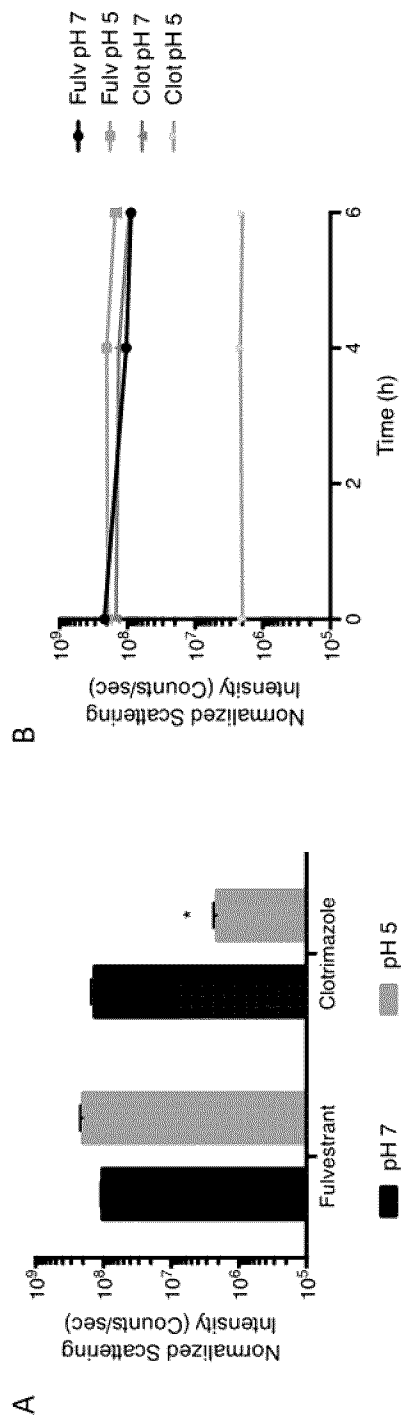


Figure 23

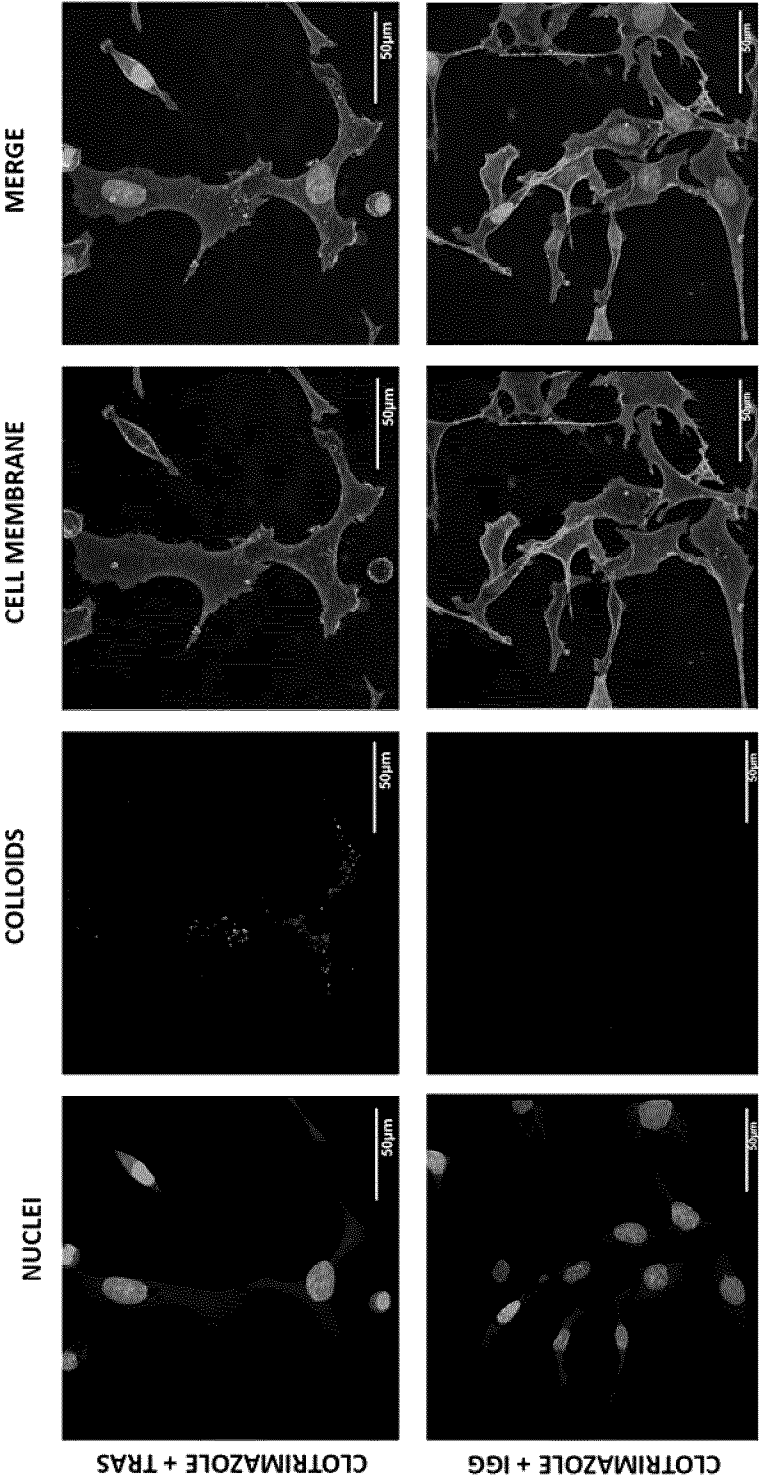


Figure 24

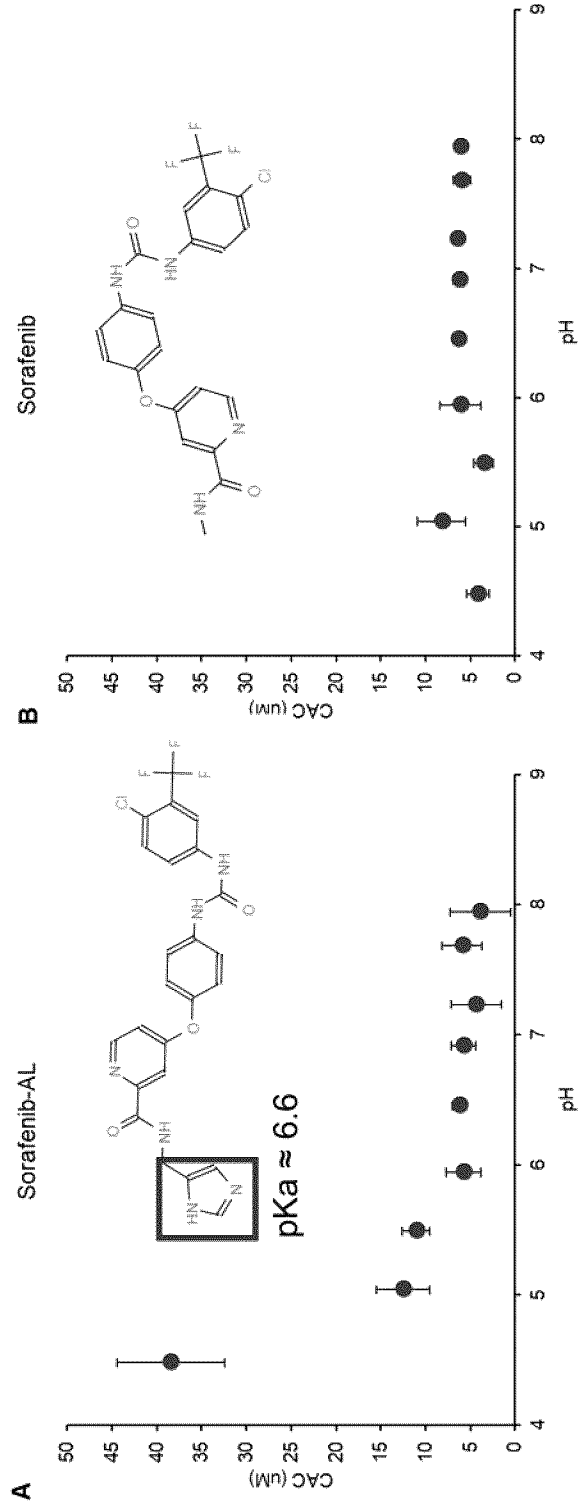


Figure 25

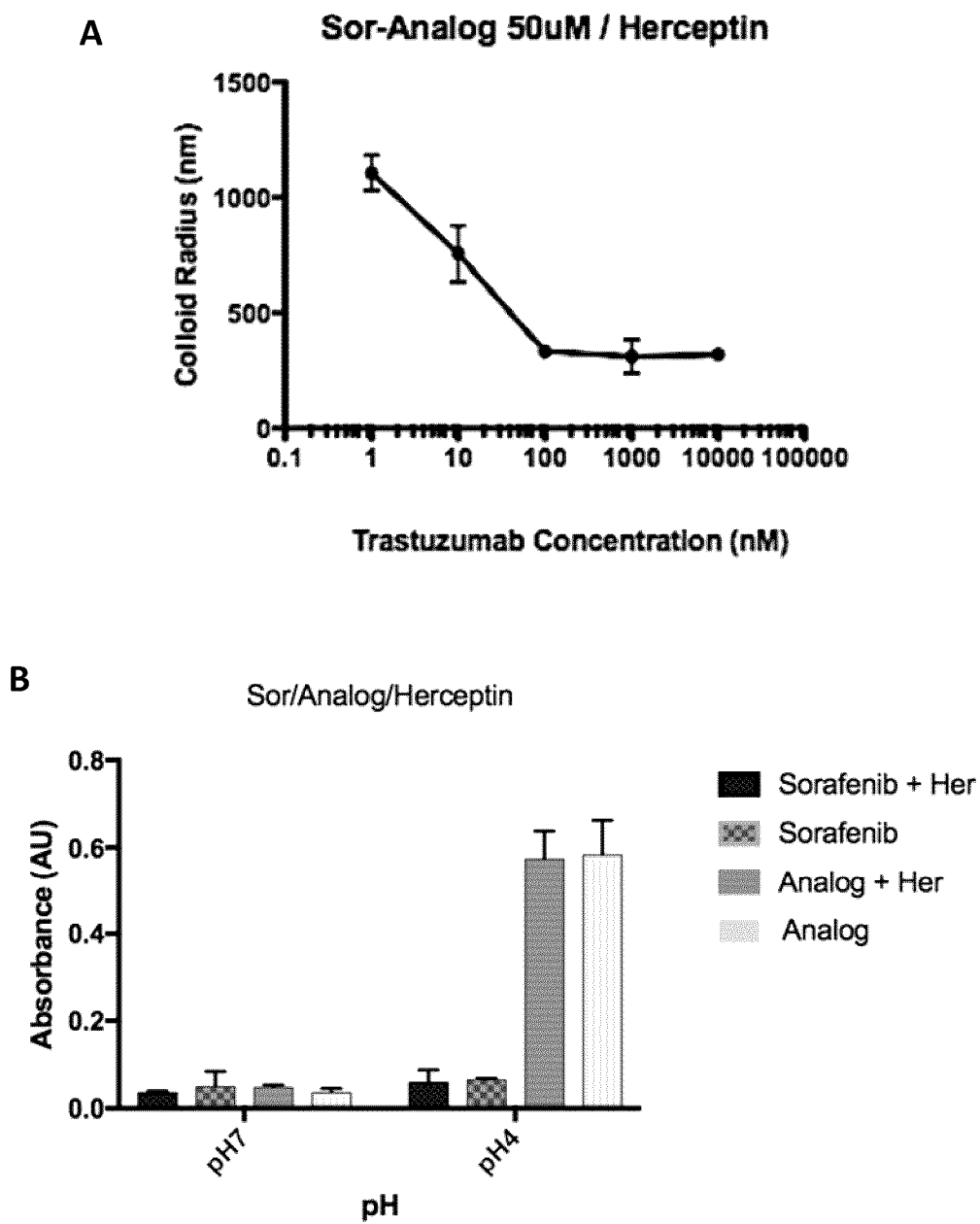


Figure 26

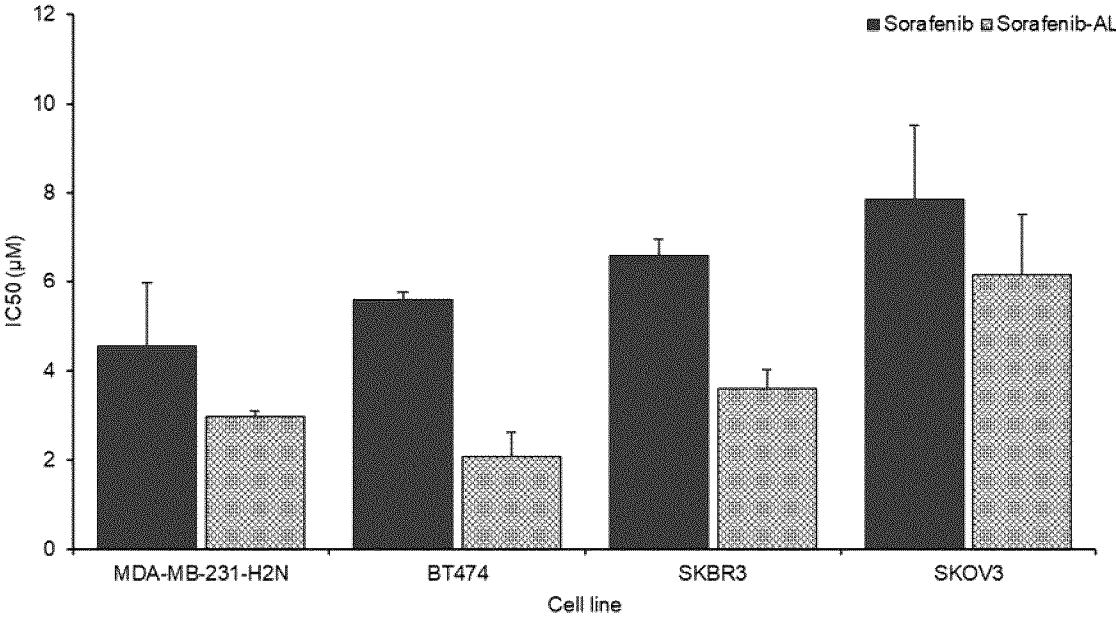


Figure 27

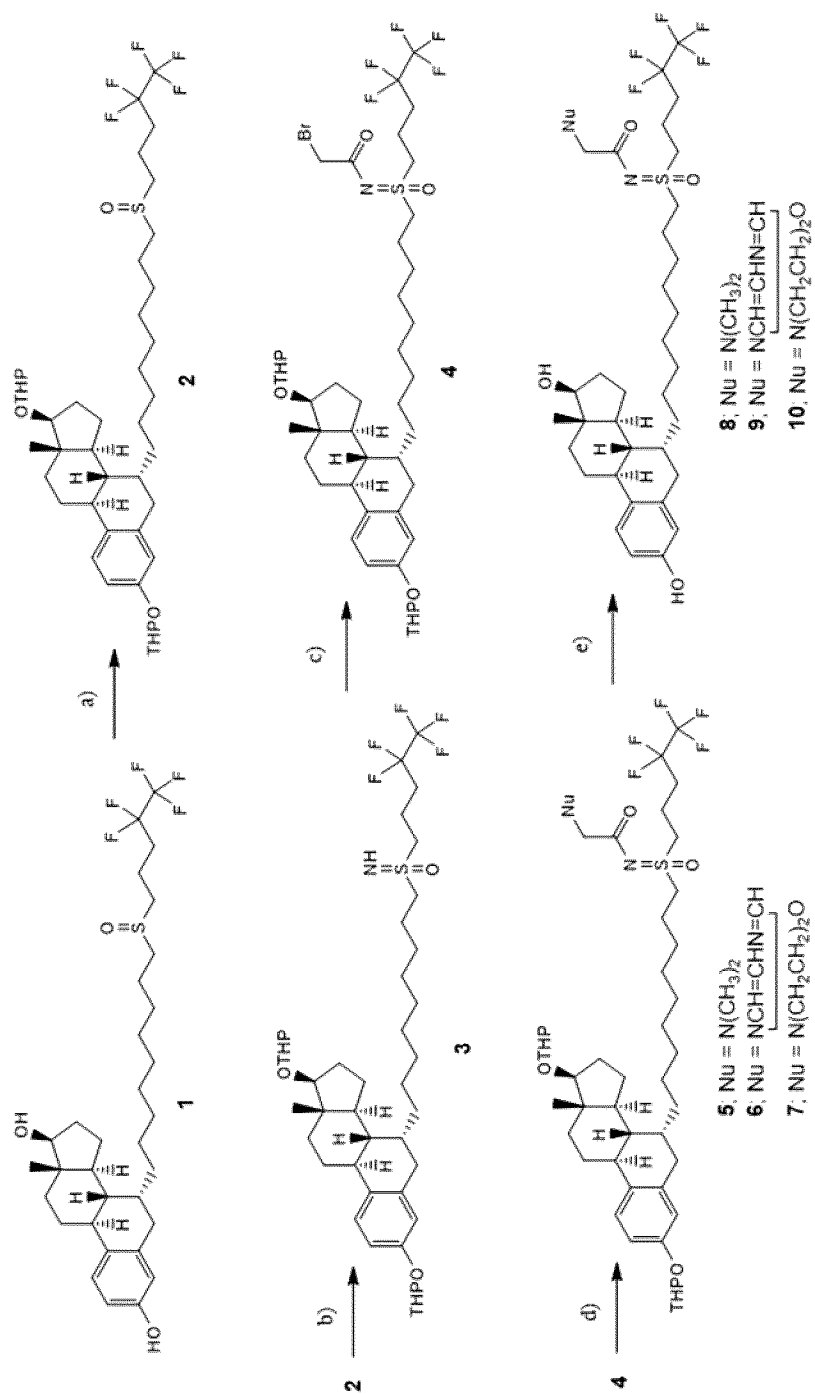


Figure 28

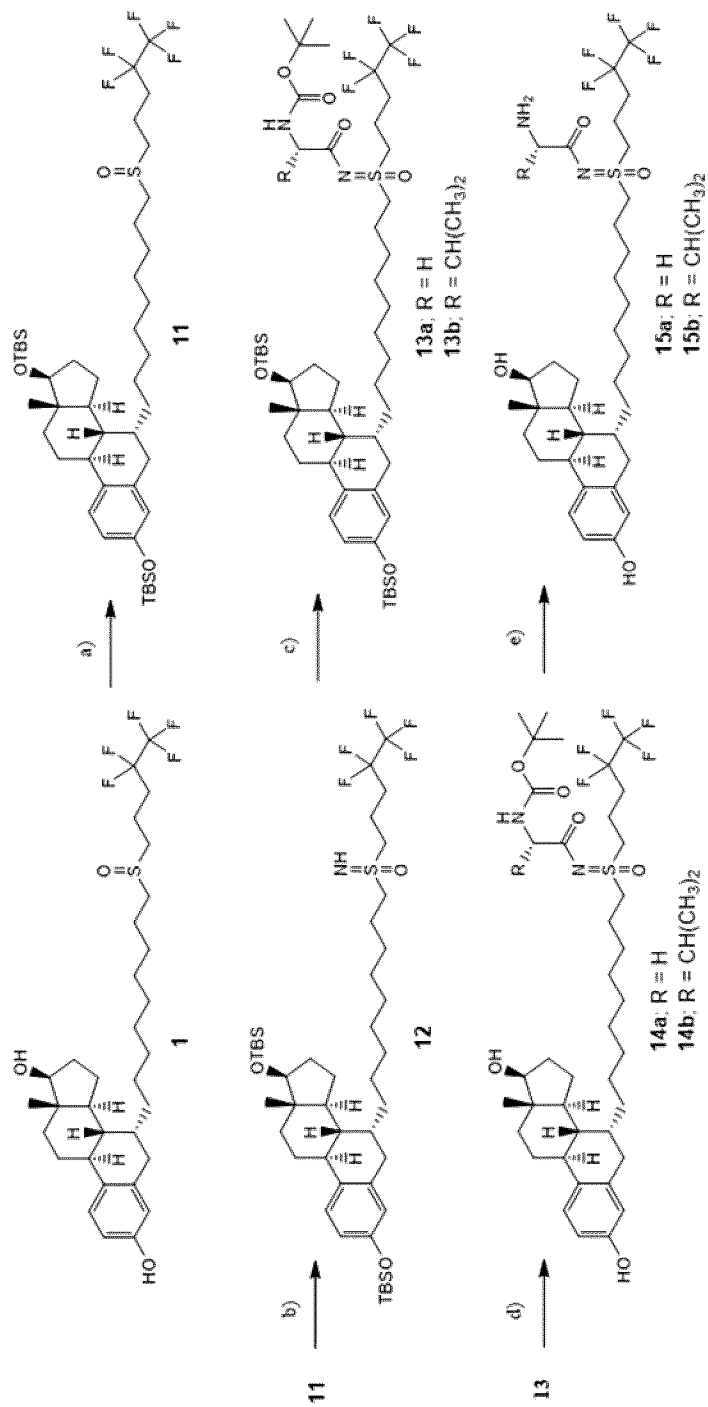


Figure 29

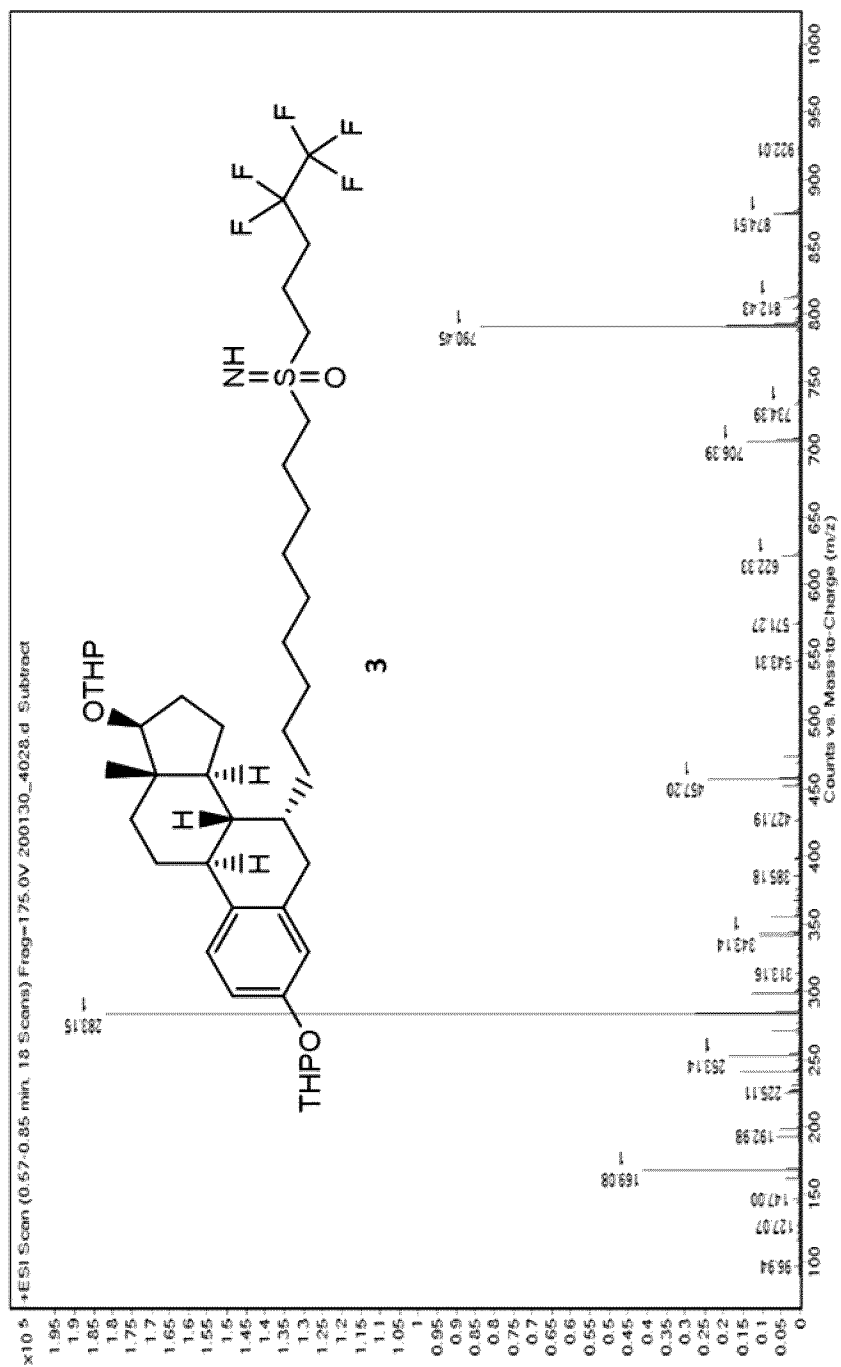
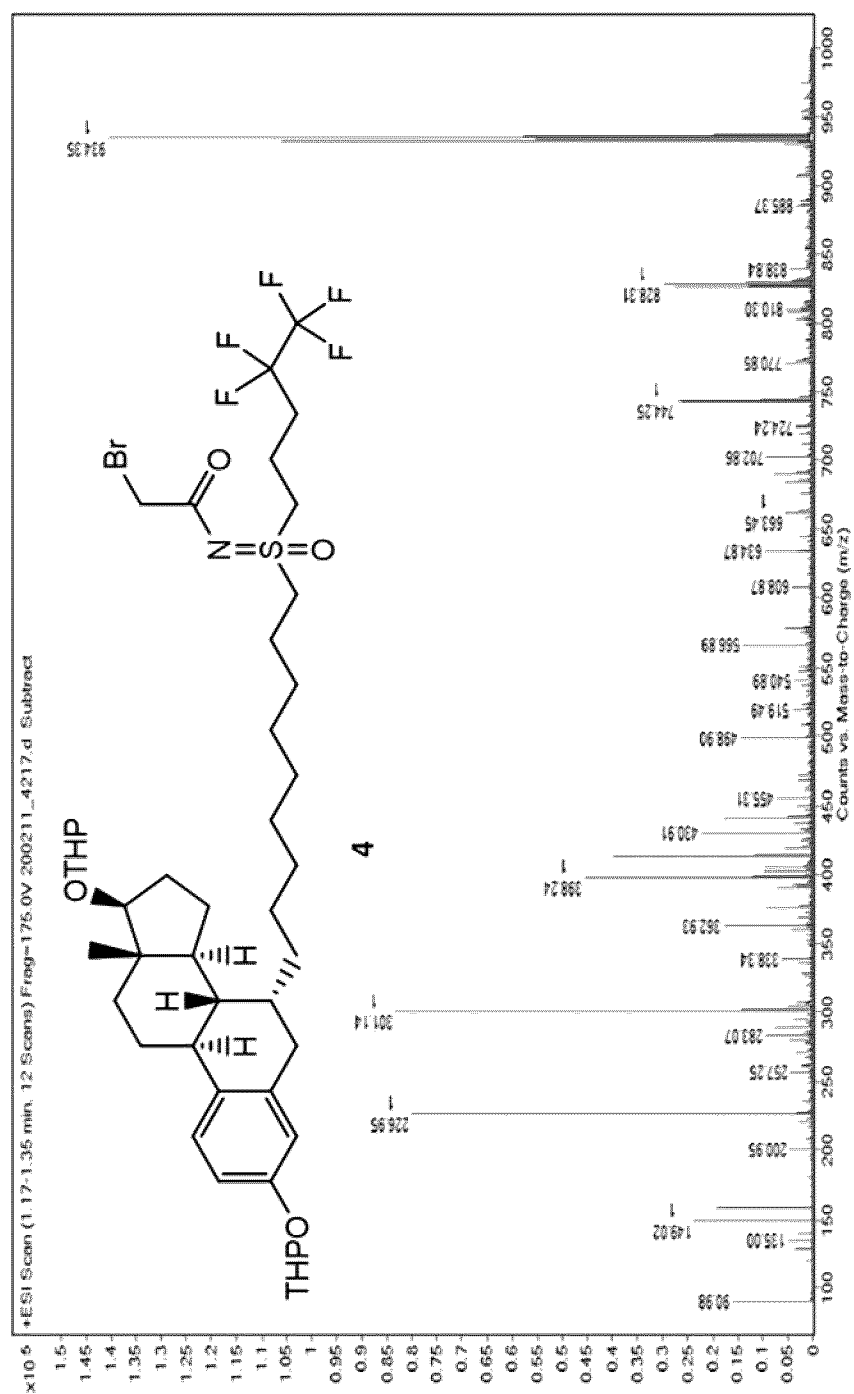


Figure 30



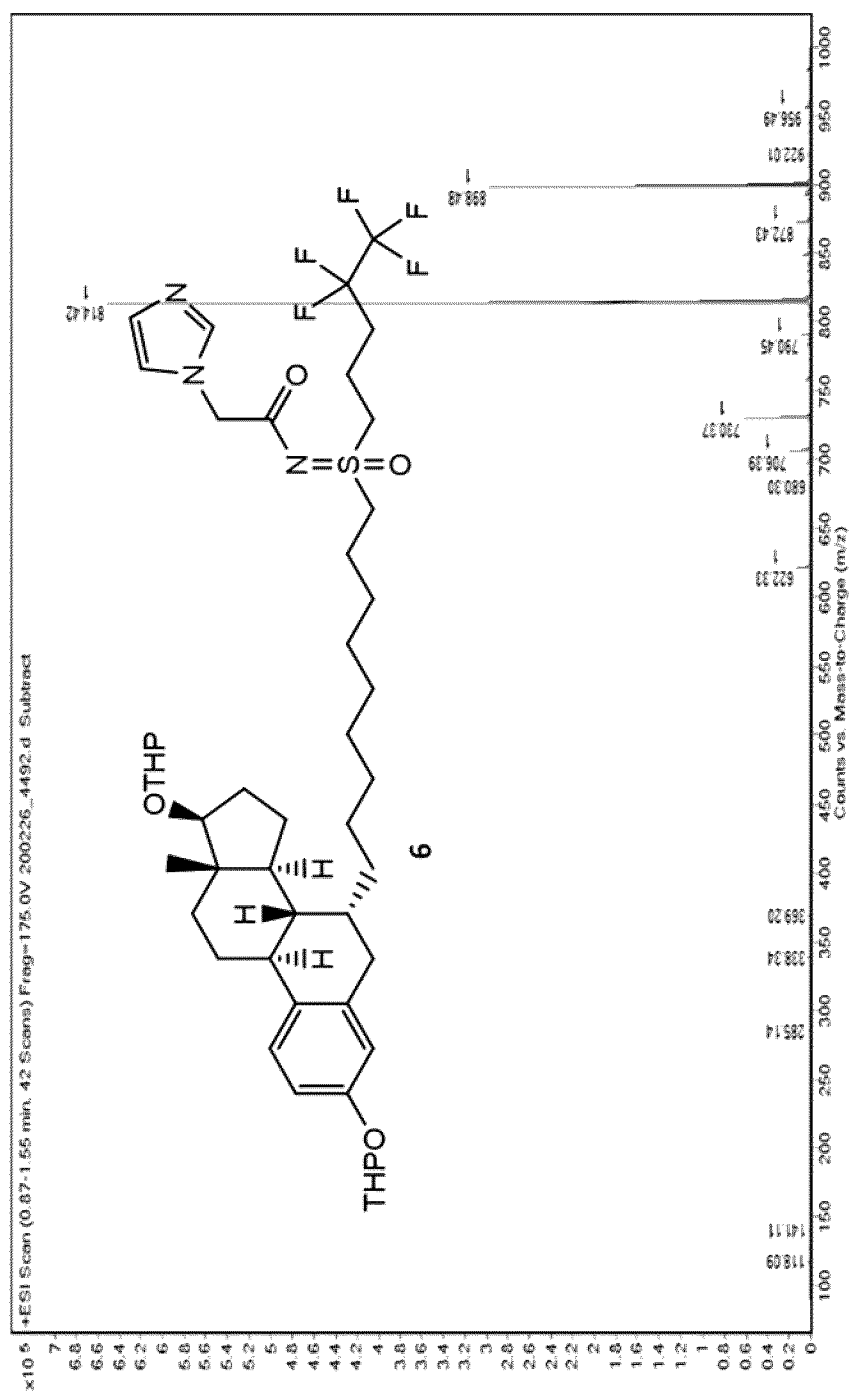
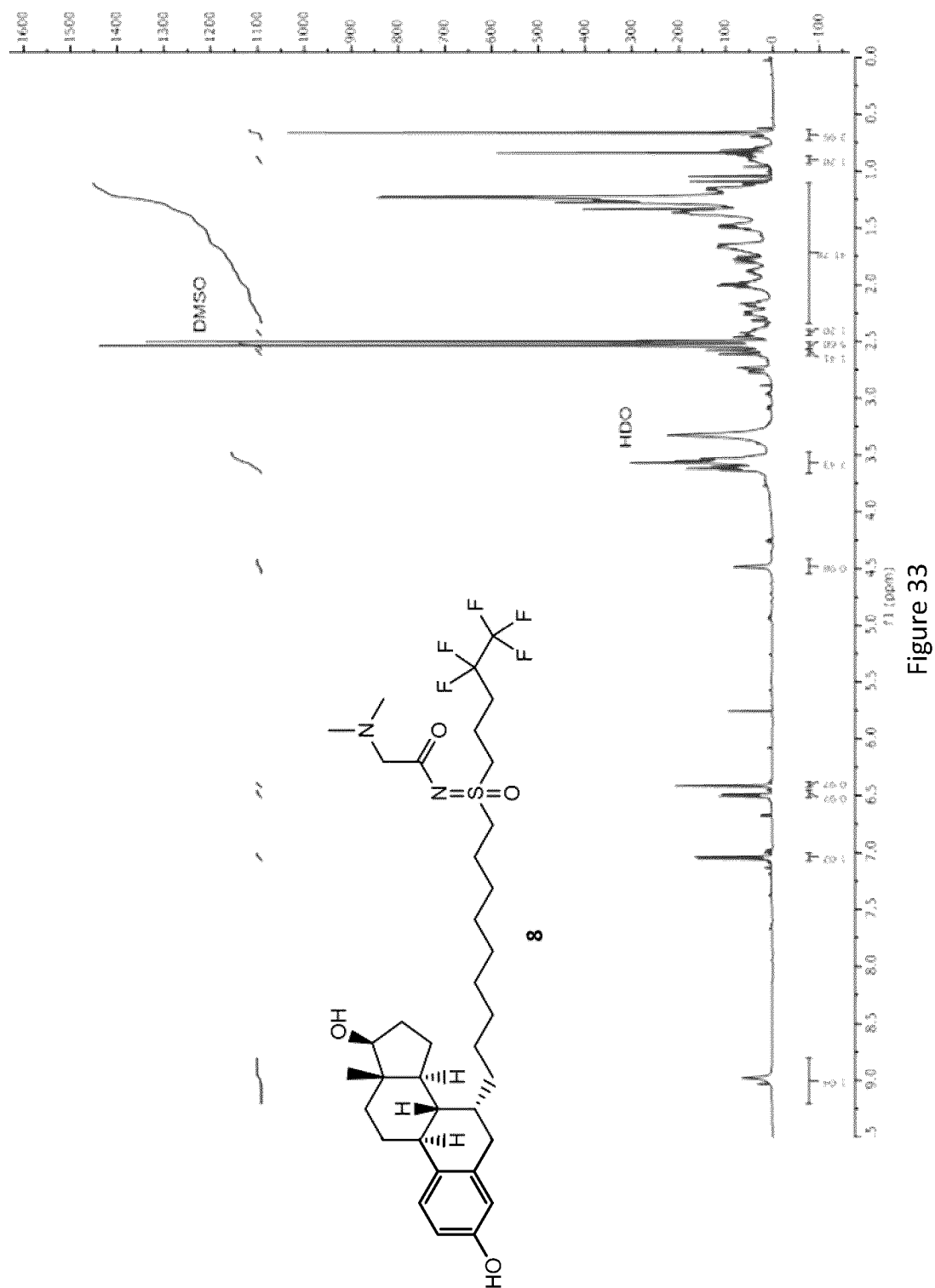


Figure 32



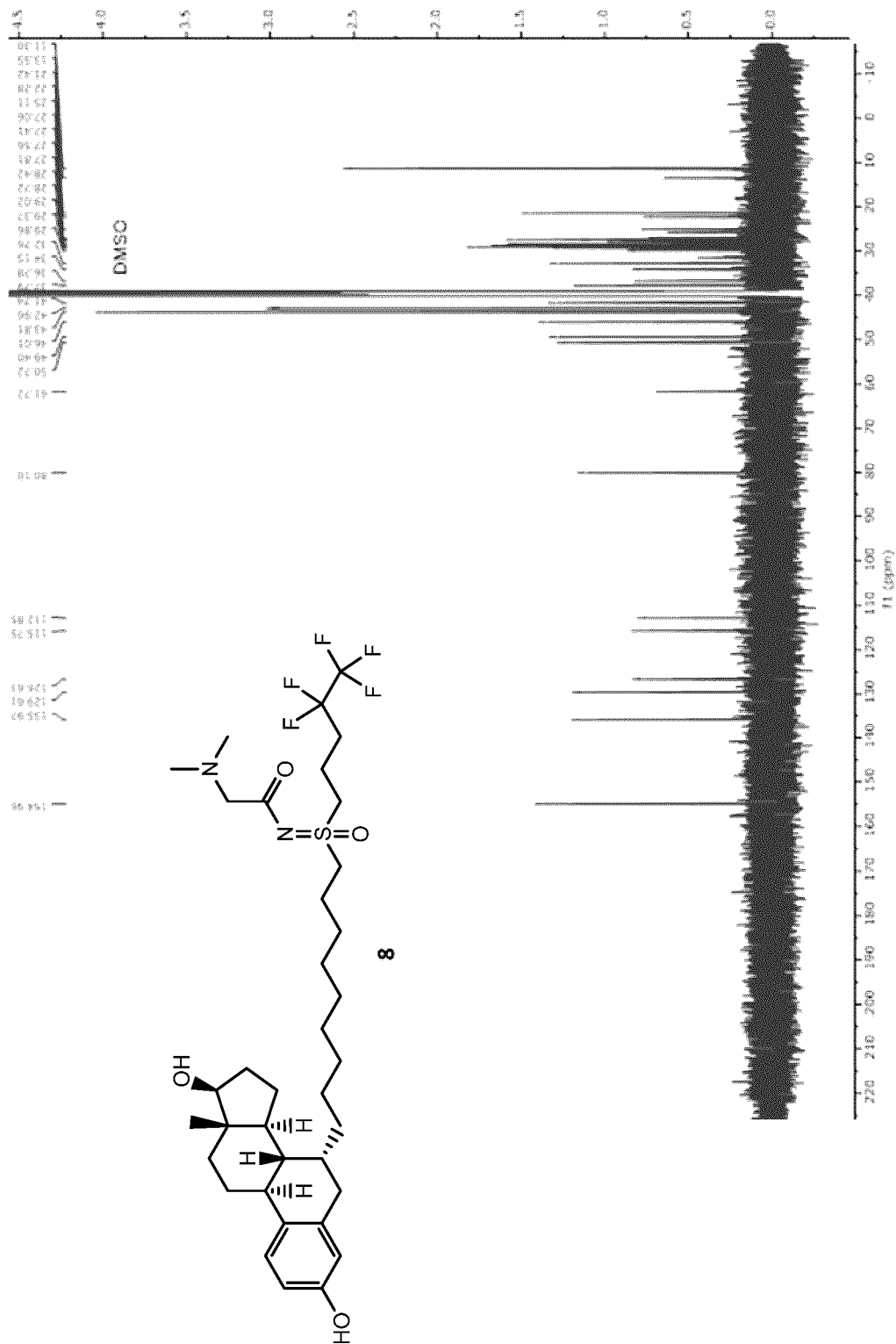


Figure 34

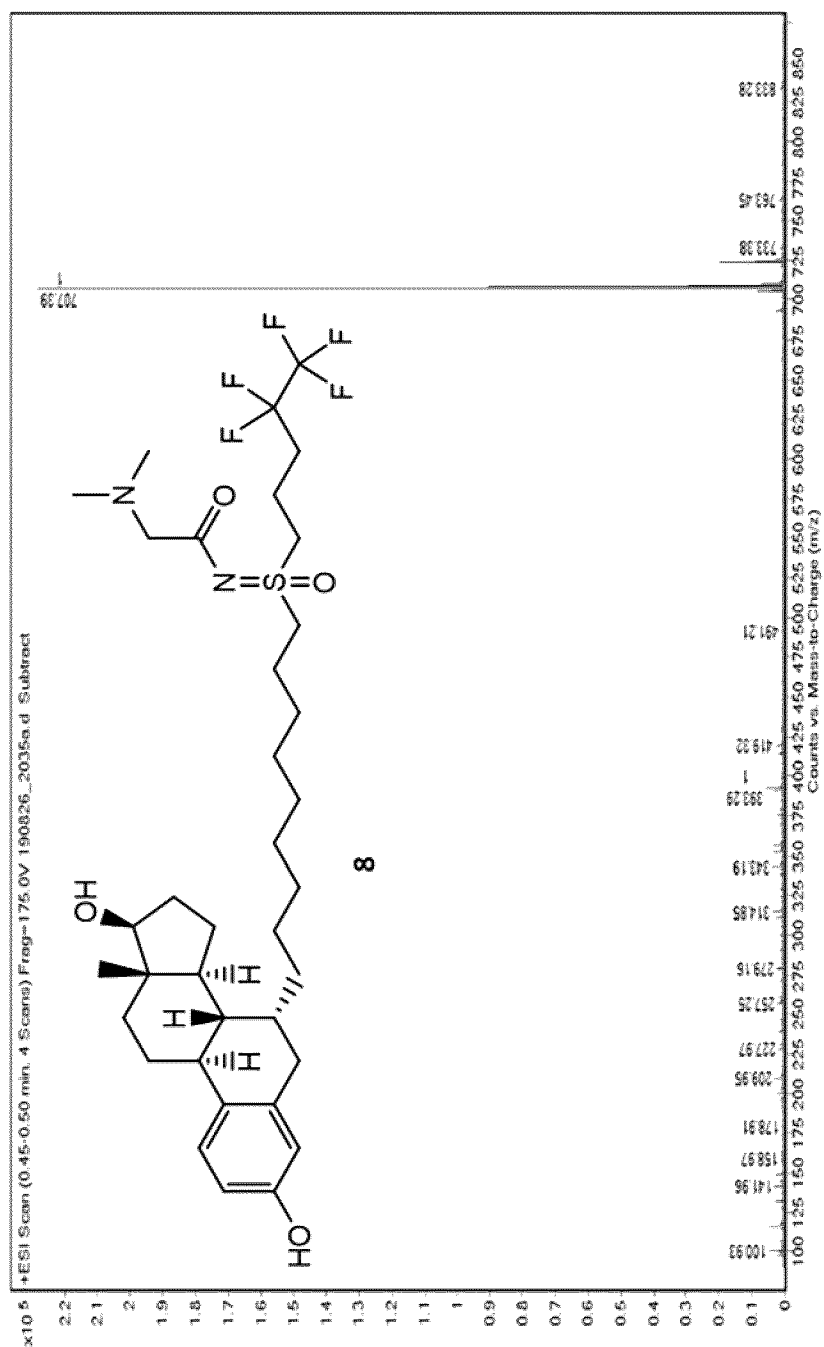


Figure 35

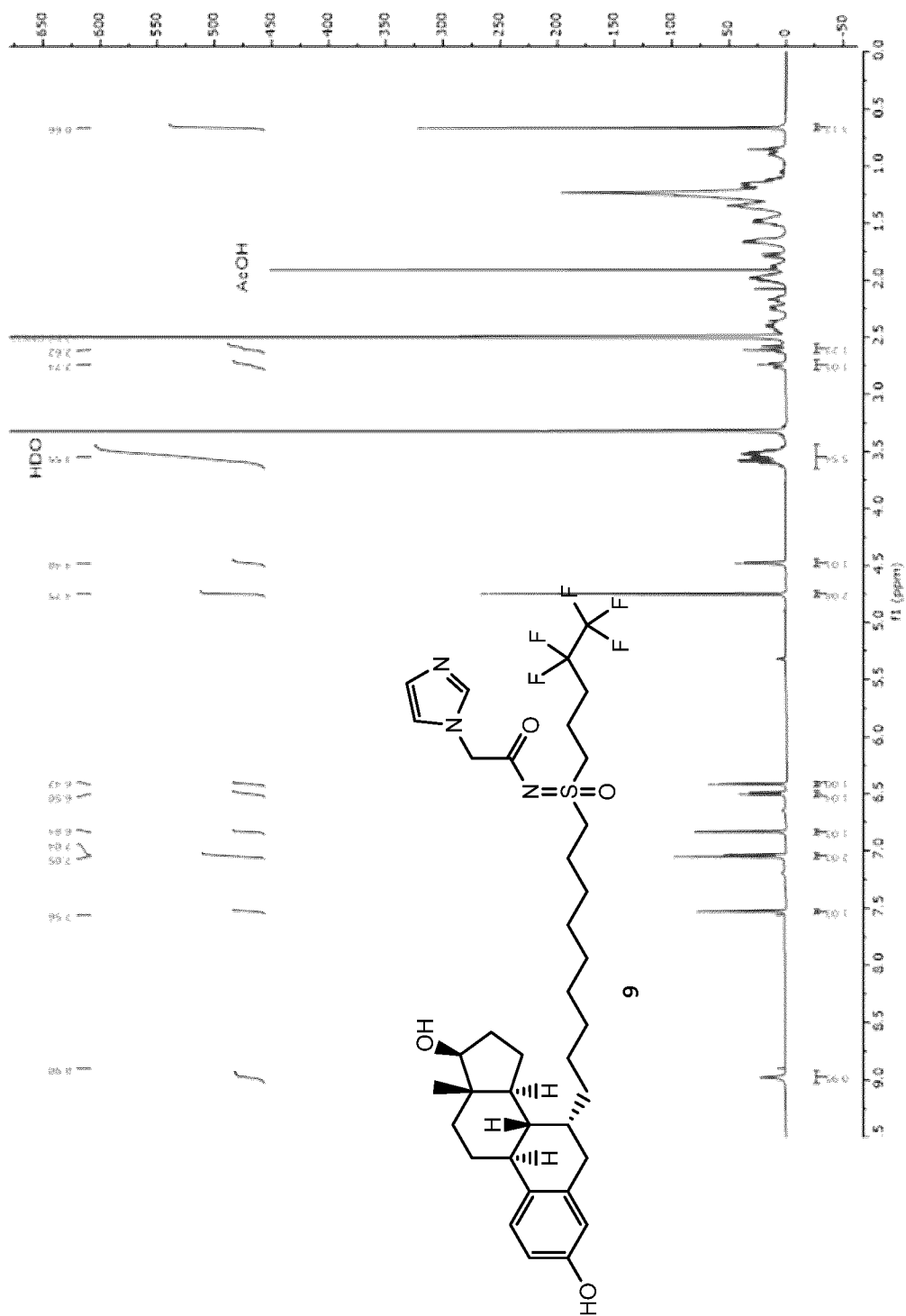


Figure 36

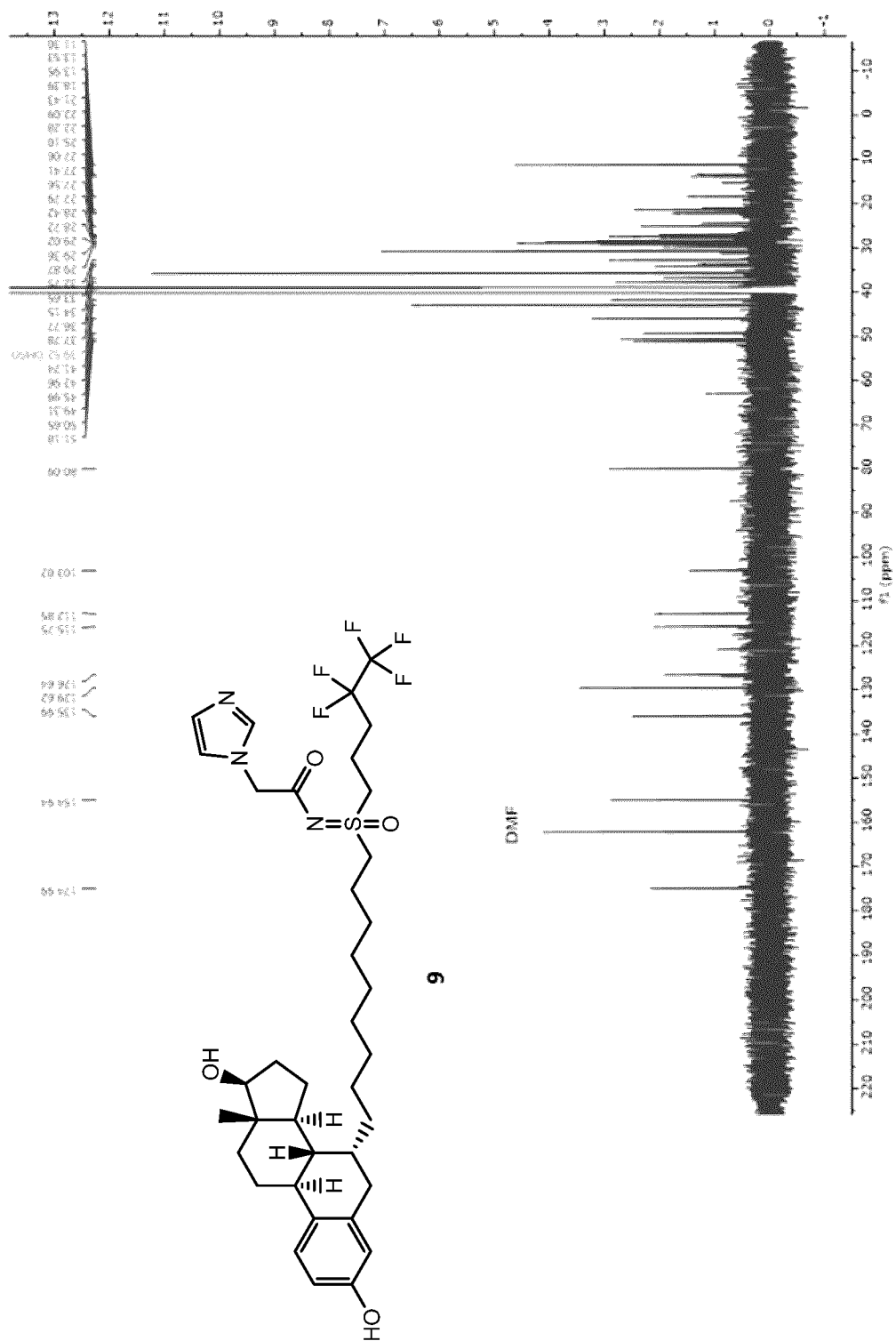


Figure 37

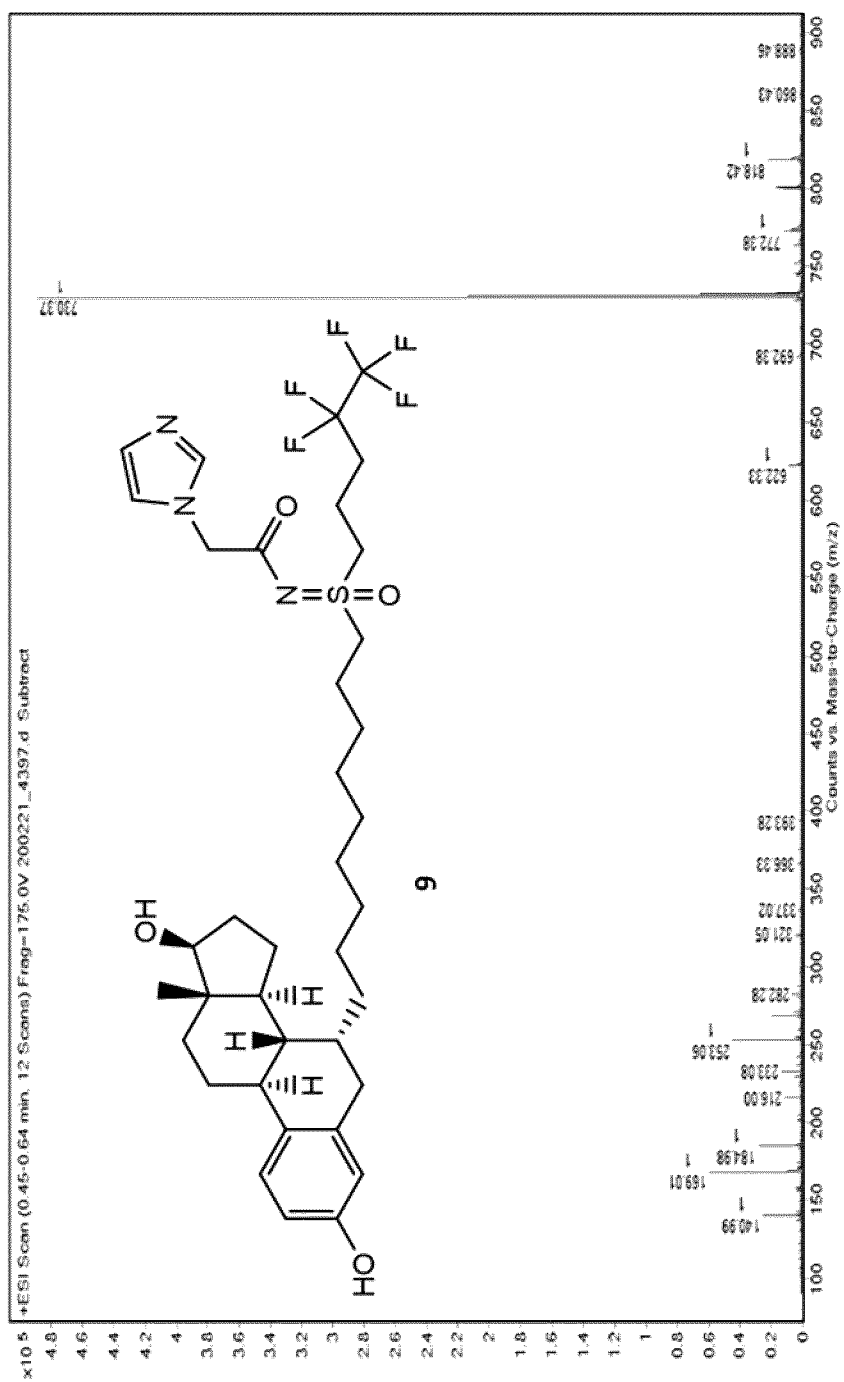


Figure 38

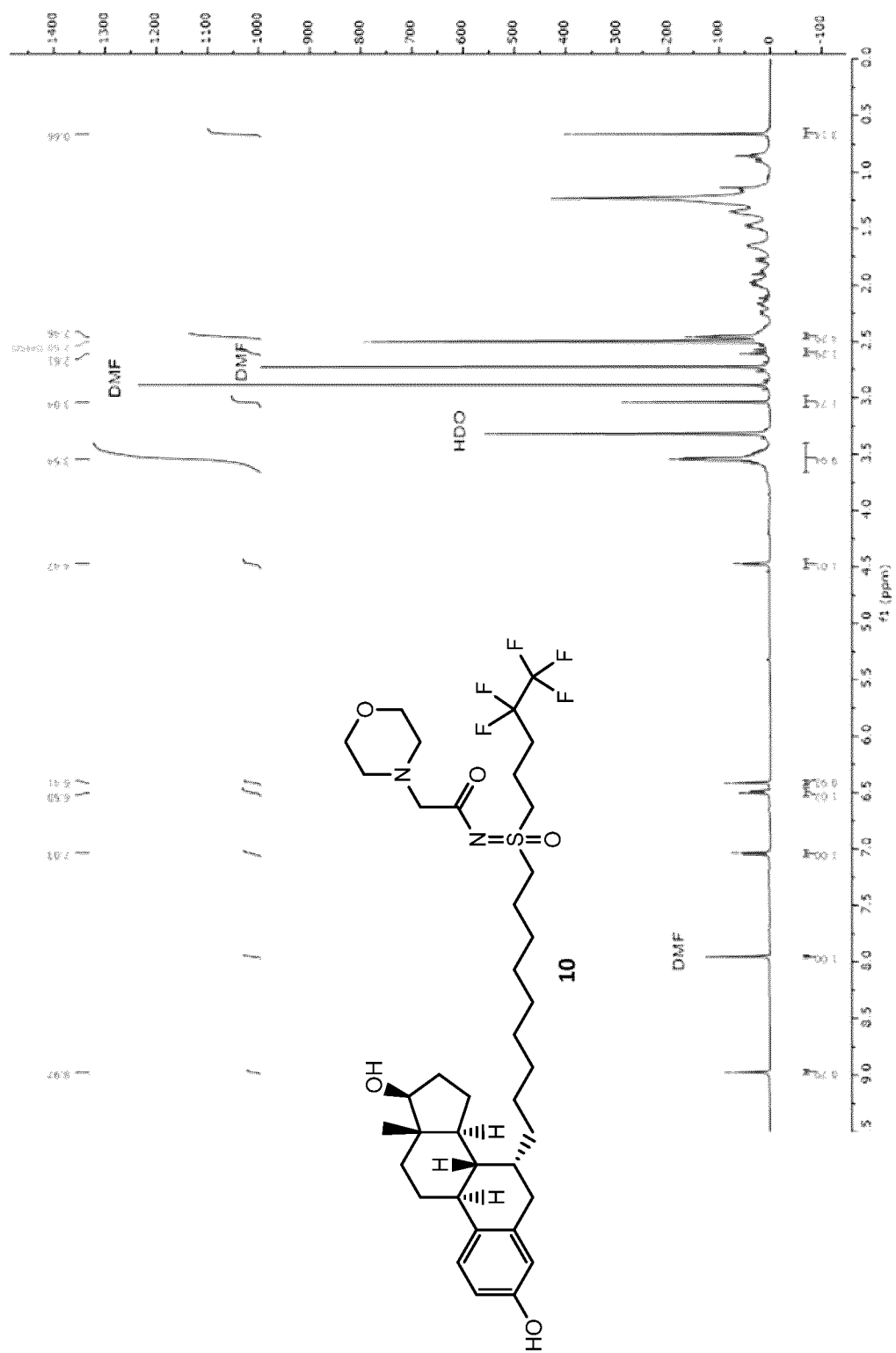


Figure 39

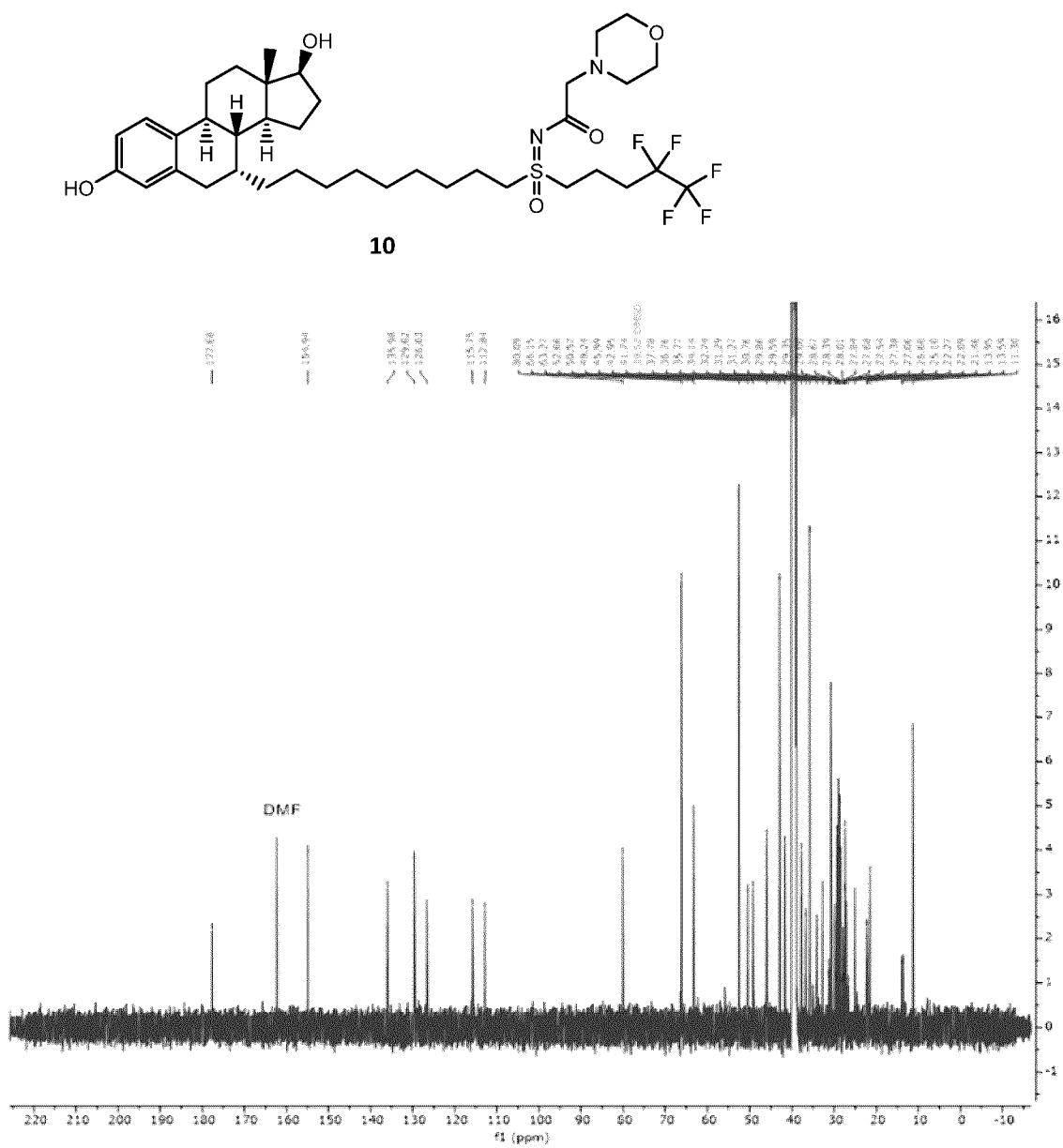


Figure 40

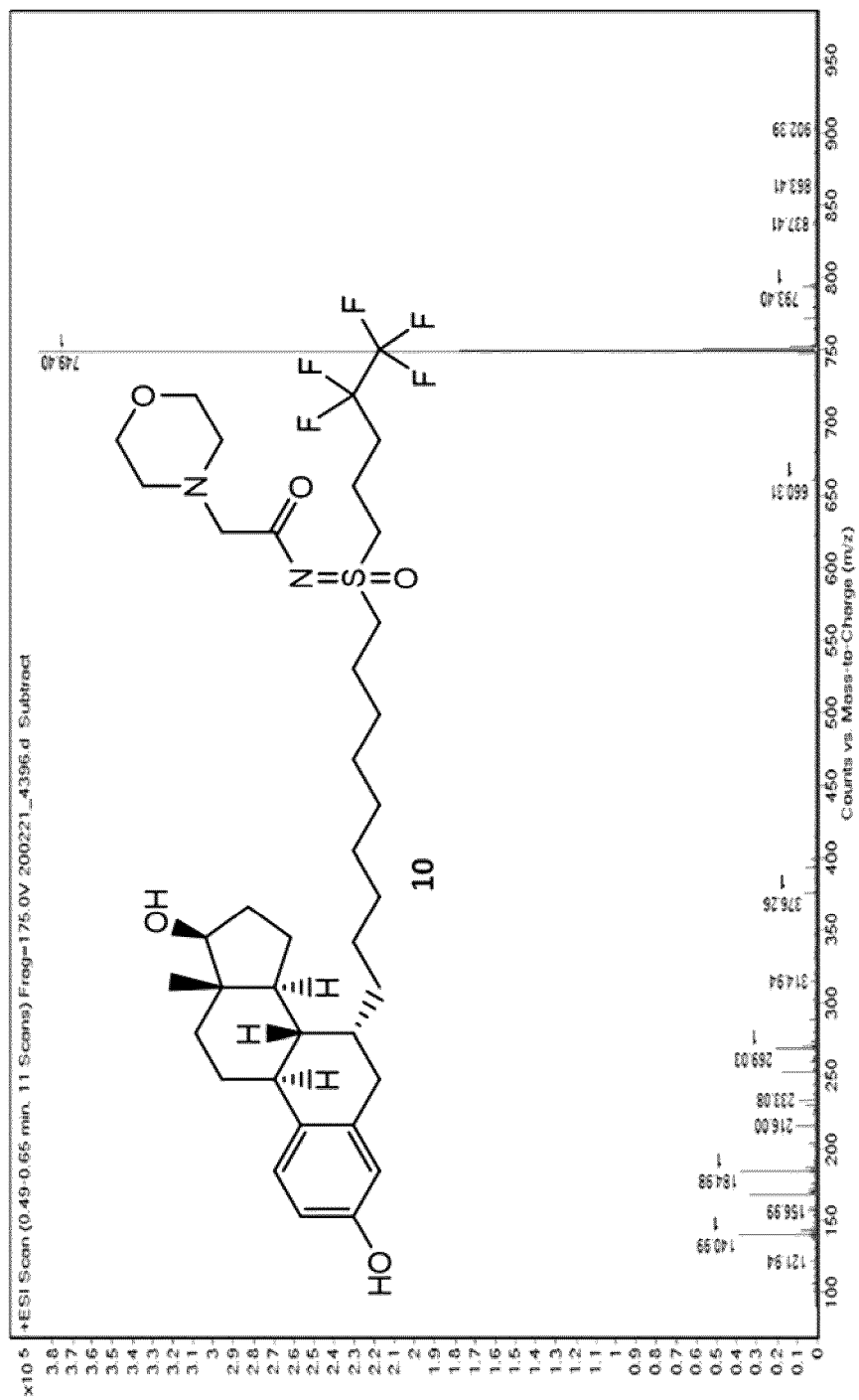


Figure 41

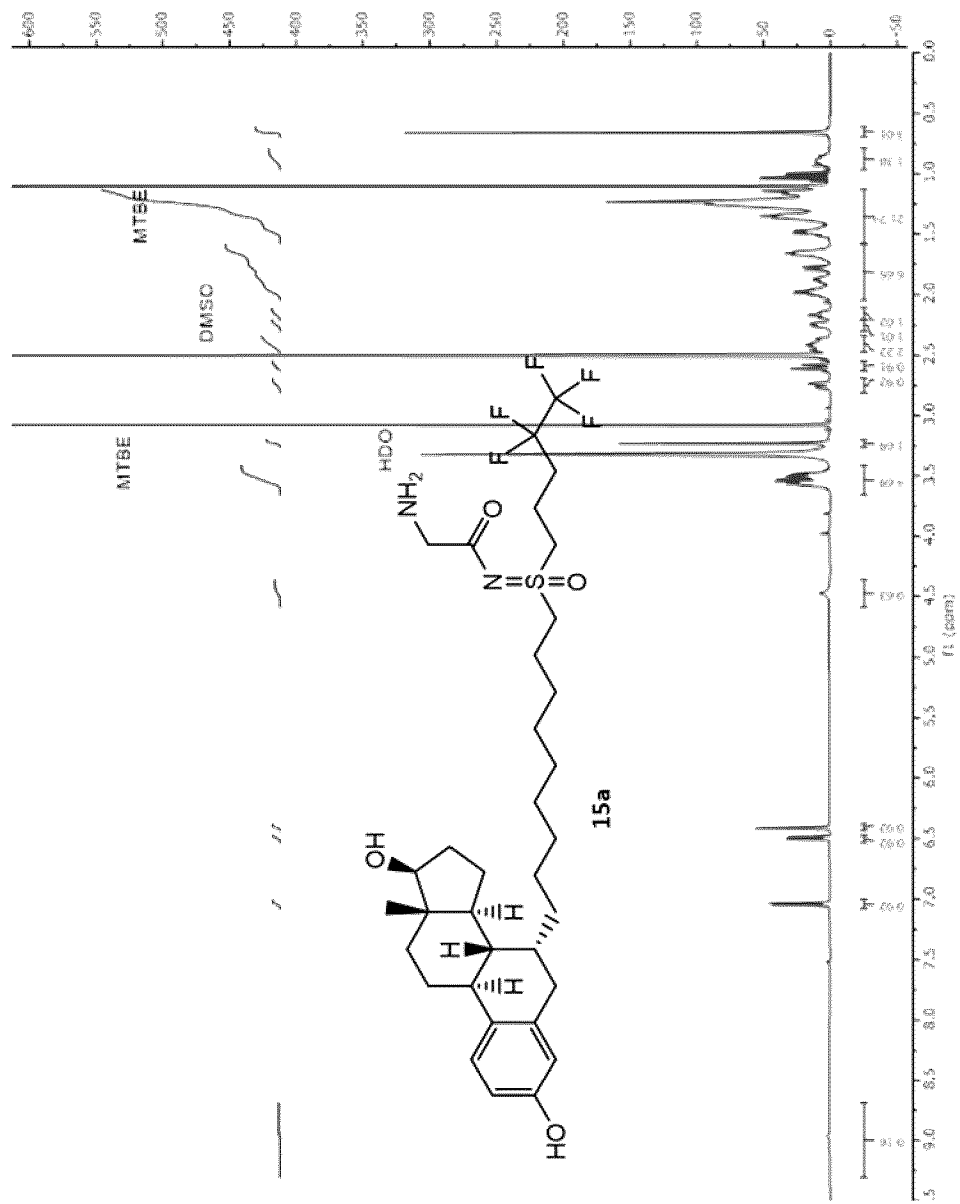


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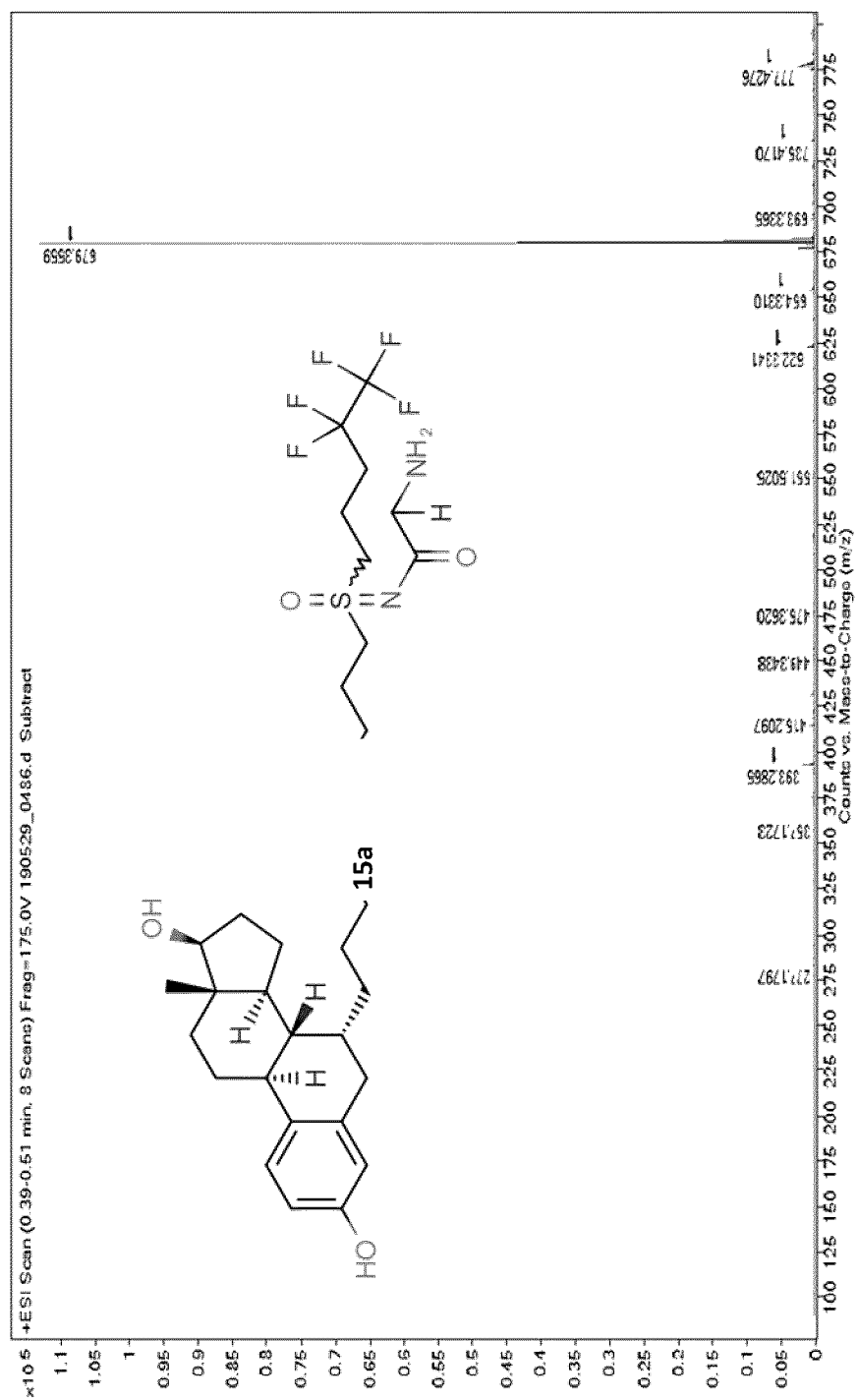


Figure 43

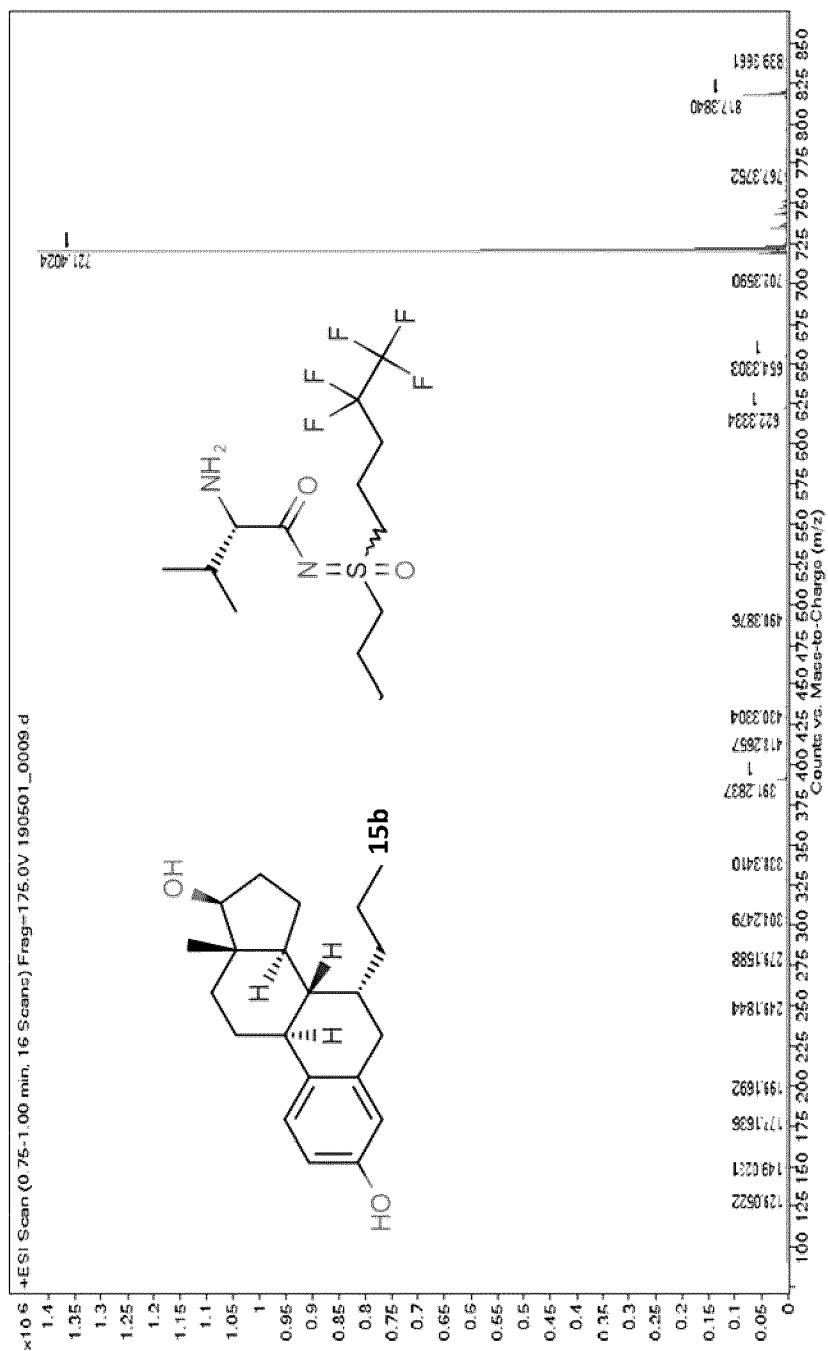


Figure 44

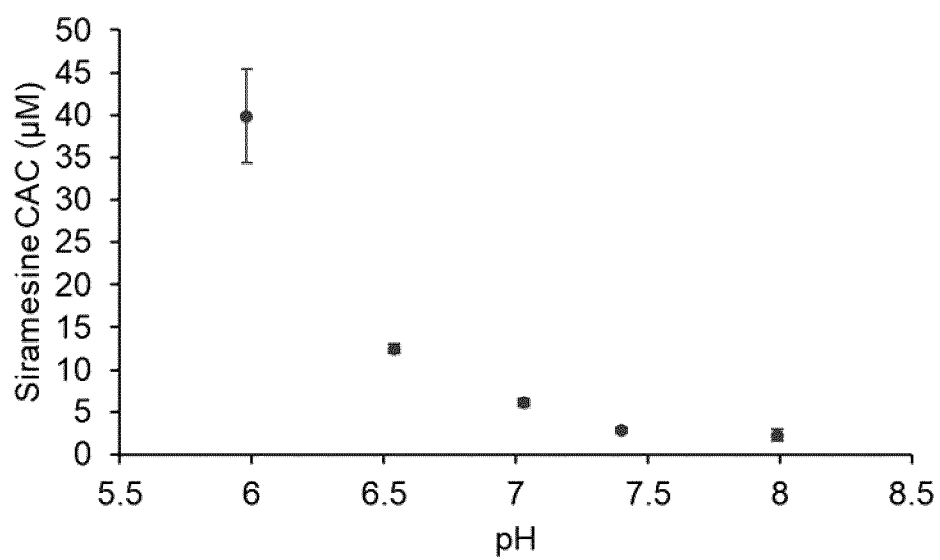


Figure 46

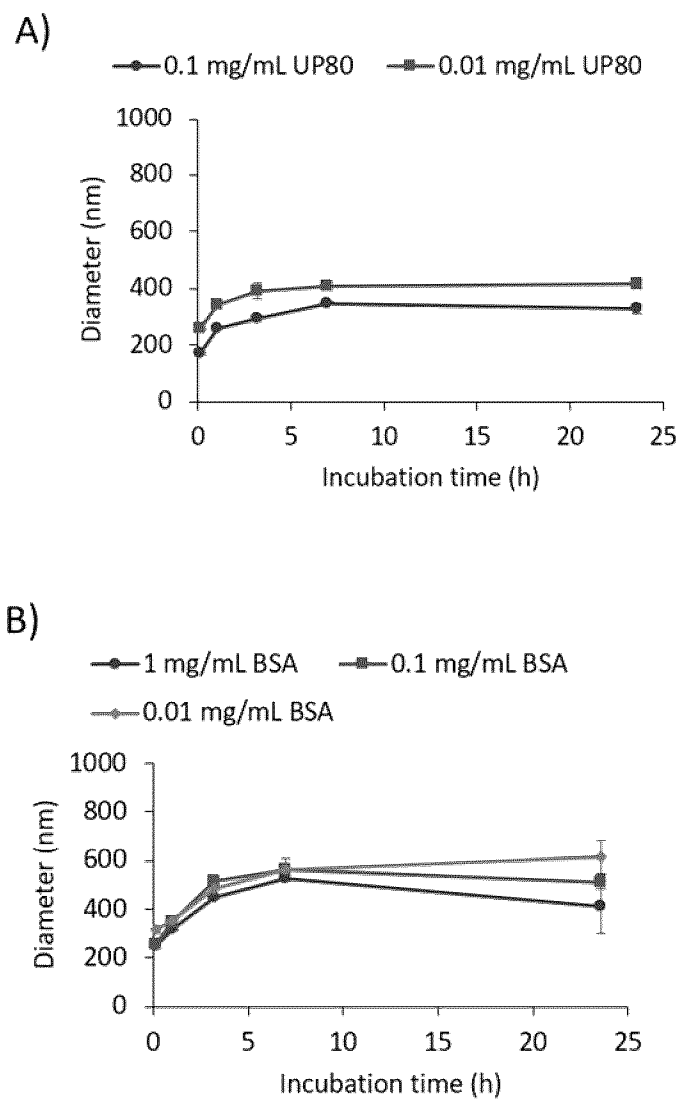


Figure 47

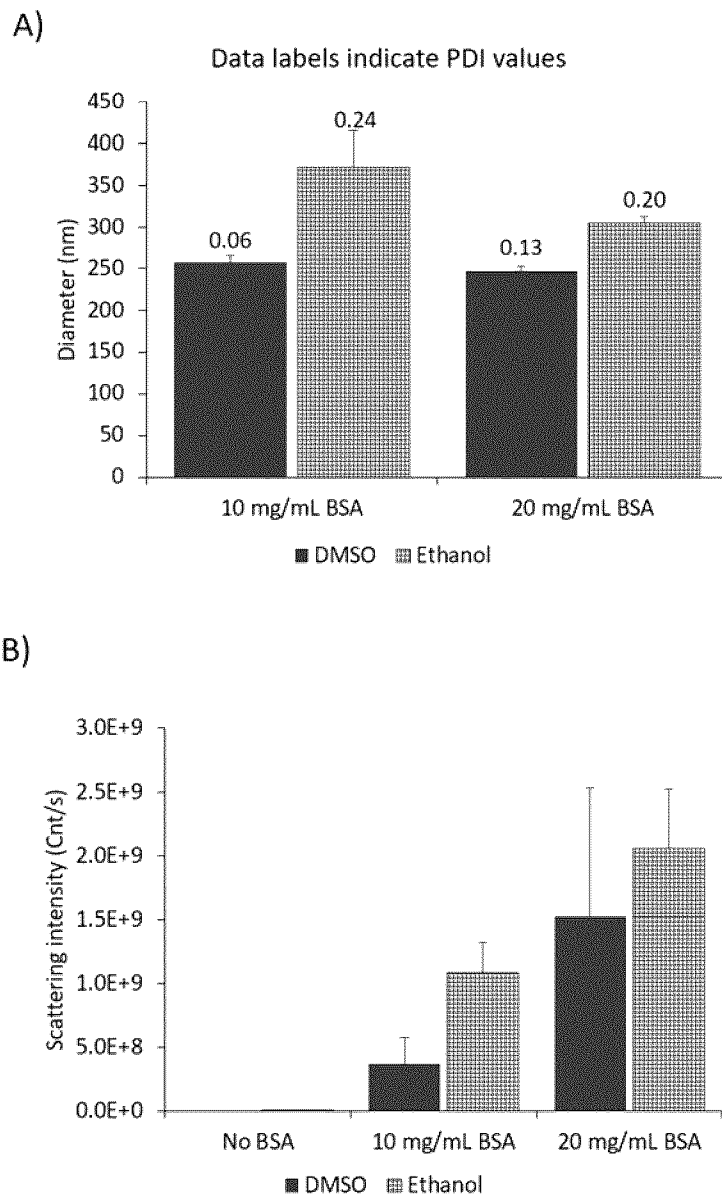


Figure 48

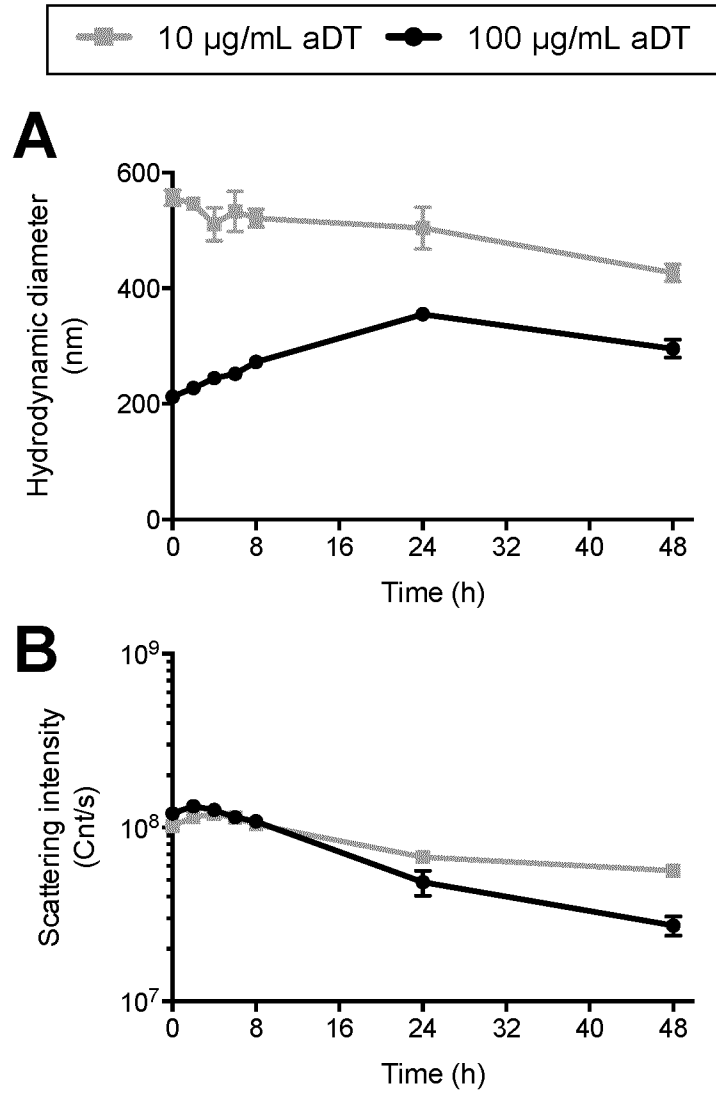


Figure 49

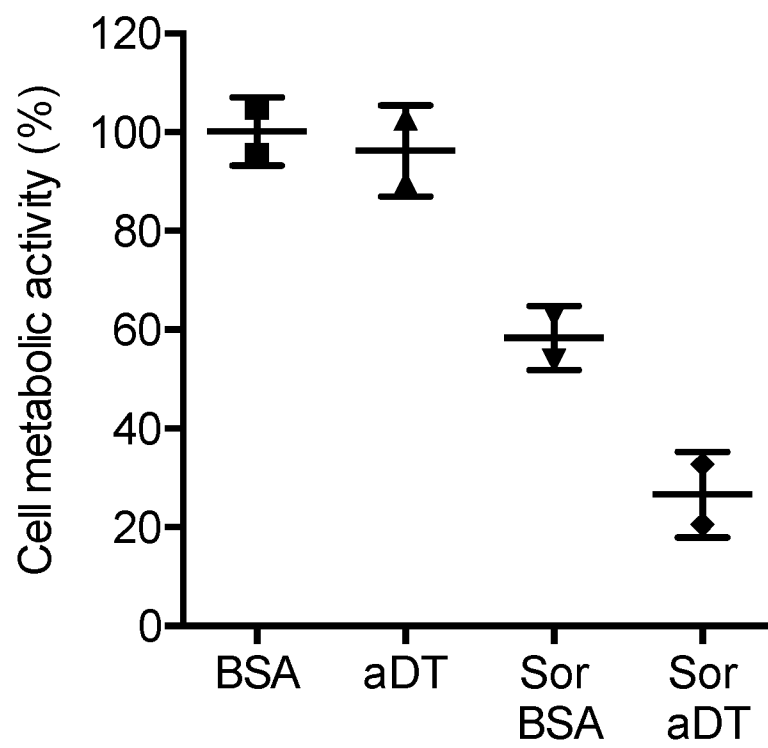


Figure 50

Table 1

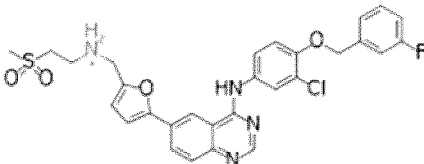
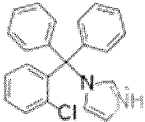
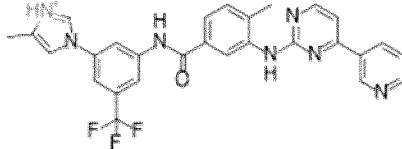
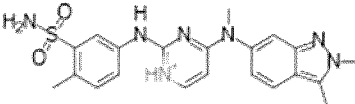
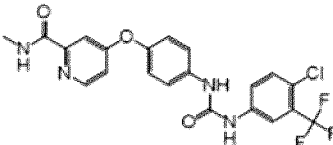
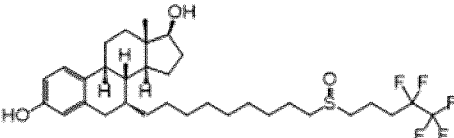
	Drug Name	Structure and protonation site	Critical aggregation concentration (μM)		pKa of DH^+
			pH 7.4	pH 5.5	
pH-Responsive	Lapatinib		1.2 ± 0.3	17 ± 3	7.2
	Clotrimazole		24 ± 1	>50	6.62
	Nilotinib		4.4 ± 1.4	15 ± 5	6.3
	Pazopanib		20 ± 4	>50	5.07
pH-Unresponsive	Sorafenib		5.9 ± 0.5	2.4 ± 1.1	2.03
	Fulvestrant		1.4 ± 0.4	1.6 ± 0.0	-0.88

Table 2

Term	Meaning	Units
Signal	Signal that is produced by colloidal particles but not by free drug (scattering or fluorescence).	Counts / s or intensity units
Signal _{colloids}	Portion of the signal that is produced by colloidal drug aggregates	Counts / s or intensity units
Signal _{BL}	Baseline signal produced by buffer and plate.	Counts / s or intensity units
H(x)	Heaviside step function. It reflects the fact that the signal only starts to rise when colloids are present. $H(x) = \begin{cases} 0, & x < 0 \\ 1, & x \geq 0 \end{cases}$	Dimensionless
C _{agg}	Concentration of aggregator present in sample.	μM
C _{agg,colloids}	Concentration of aggregator present in the colloidal state	μM
CAC _{agg}	Critical aggregation concentration of the aggregator under assay conditions.	μM
m	Amount of signal produced per amount of aggregator. More aggregator means more particles, which means more scattering or fluorescence.	Signal units / μM
n	Qualitative correction factor that accounts for non-linear signal to concentration relationship, especially in scattering-based assays. This factor is necessary for a high-quality fit because differences in colloid size can result in deviations from the expected straight line. Since these deviations are relatively small, this factor is usually between 0.8 and 1.2.	Dimensionless

Table 3

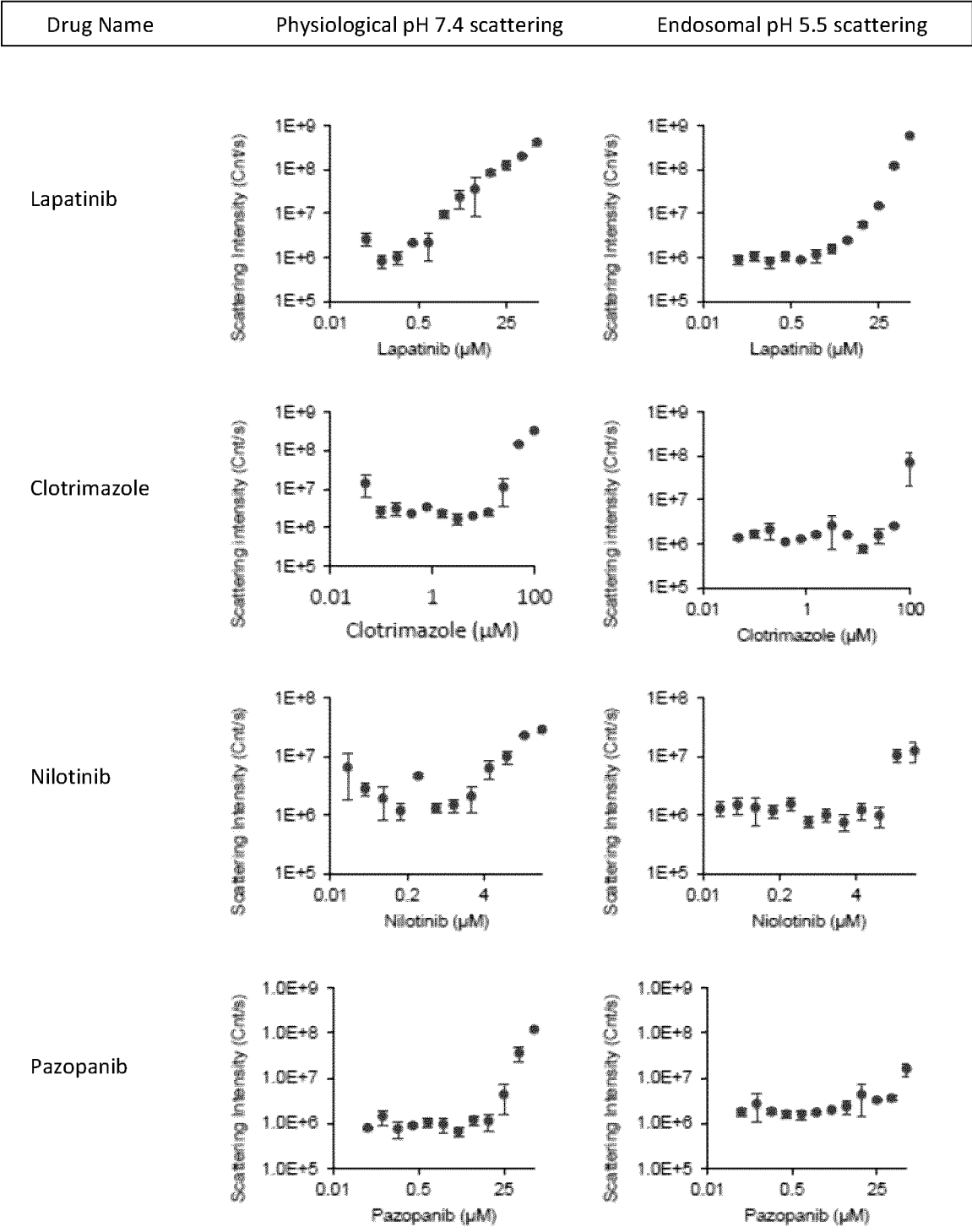


Table 3 cont.

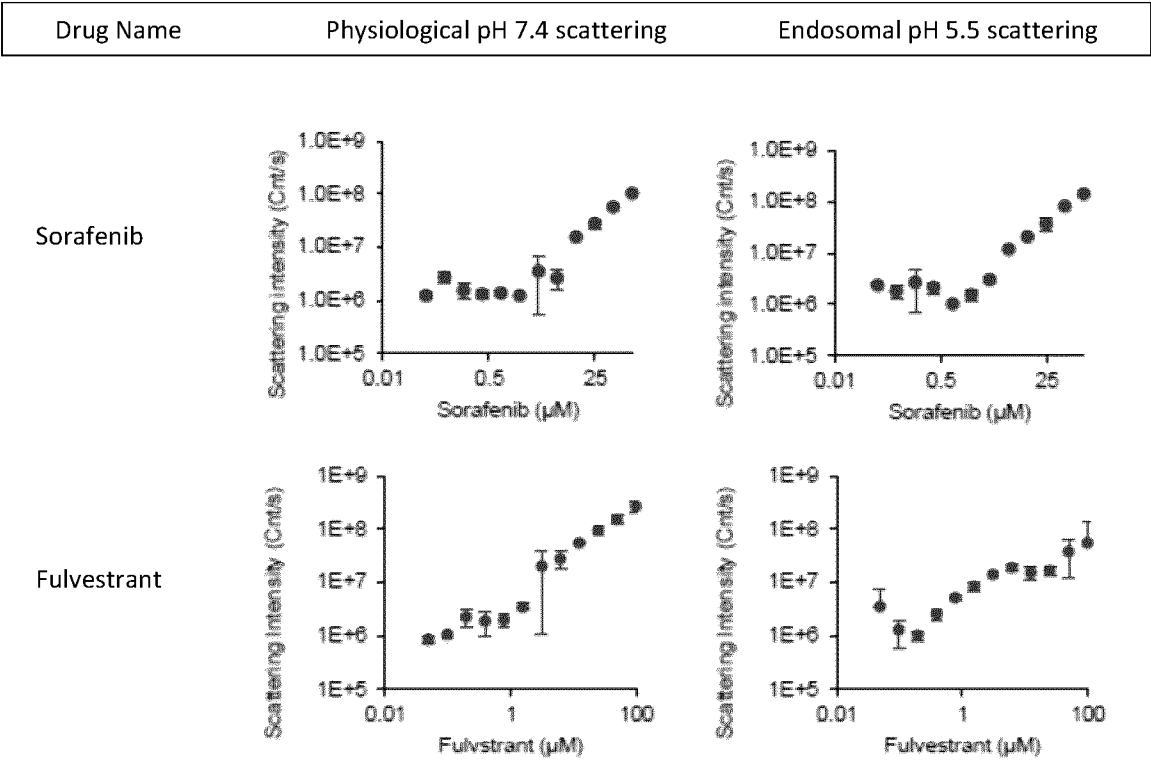


Table 4

Name	Lapatinib μM	Fulvestrant μM	PLAC-PEG mg/mL	Transferrin mg/mL
Lapatinib/fulvestrant, transferrin	50	150	0	0.1
Lapatinib/fulvestrant, PLAC-PEG	50	150	0.04	0
Fulvestrant, transferrin	0	150	0	0.1
Fulvestrant, PLAC-PEG	0	150	0.04	0
No drug, transferrin	0	0	0	0.1
No drug, PLAC-PEG	0	0	0.04	0
Blank	0	0	0	0

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2020/050845

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 9/10** (2006.01), **A61K 31/4174** (2006.01), **A61K 31/438** (2006.01), **A61K 31/506** (2006.01),
A61K 31/517 (2006.01), **A61K 31/565** (2006.01) (more IPCs on the last page)

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)
A61K (2006.01), **C07D** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Scopus, Canadian Patent Database, Questel Orbit, Google: keywords: colloid, colloidal, aggregate, acid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	GANESH, A.N. ET AL., "A New Spin on Antibody-Drug Conjugates: Trastuzumab-Fulvestrant Colloidal Drug Aggregates Target HER2-Positive Cells", <i>ACS Appl. Mater. Interfaces</i> (2017), 9:12195-12202 See entire document; especially results and discussion.	1-10, 13-18, 27, 28; 12
X, Y	GANESH, A.N. ET AL., "Leveraging Colloidal Aggregation for Drug-Rich Nanoparticle Formulations", <i>Mol. Pharmaceutics</i> (2017), 14:1852-1860 See entire document; especially colloid formation, results and discussion.	1-9, 11, 13, 14, 22-25, 27, 28; 12
Y	OWEN, S.C. ET AL., "Colloidal Aggregation Affects the Efficacy of Anticancer Drugs in Cell Culture", <i>ACS Chem. Biol.</i> (2012), 7:1429-1435 See entire document.	12

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* "A" "D" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance document cited by the applicant in the international application earlier application or patent but published on or after the international filing date 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) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	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 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 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
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2020/050845

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GANESH, A.N. ET AL., "Colloidal aggregation: From screening nuisance to formulation nuance", <i>Nano Today</i> (2018), 19:188-200 See entire document.	1-28
P, X	DONDERS, E.N. ET AL., "Triggered Release Enhances the Cytotoxicity of Stable Colloidal Drug Aggregates", <i>ACS Chem. Biol.</i> (25 June 2019; 25-06-2019), 14:1507-1514 See entire document	1-28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2020/050845

A61K 47/10 (2017.01), *A61K 47/28* (2006.01), *A61K 47/30* (2006.01), *A61K 47/42* (2017.01),
C07D 233/62 (2006.01), *C07D 239/94* (2006.01), *C07D 401/14* (2006.01), *C07D 403/12* (2006.01),
C07D 491/107 (2006.01)