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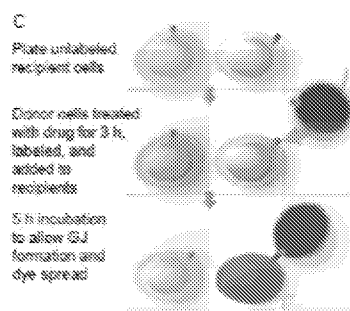
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(54) Title: INHIBITING CONNEXIN 46 TO TREAT GLIOBLASTOMA AND OTHER CONDITIONS

FIG. 3



(57) Abstract: Provided herein are compositions, systems, kits, and methods for treating a disease or condition by administering to a subject an agent that inhibits connexin 46 (Cx46) (e.g., ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine analog or derivative). In certain embodiments, the agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37 (e.g., in a human subject). In certain embodiments, the disease or condition is selected from brain cancer (e.g., glioblastoma), deafness, cataracts, or a skin disease.

INHIBITING CONNEXIN 46 TO TREAT GLIOBLASTOMA AND OTHER CONDITIONS

The present application claims priority to U.S. Provisional application 62/560,251
5 filed September 19, 2017, and Provisional application 62/644,687 filed March 19, 2018, both
of which are herein incorporated by reference in their entireties.

STATEMENT REGARDING FEDERAL FUNDING

This invention was made with government support under grant number NS089641
10 awarded by the National Institutes of Health. The government has certain rights in the
invention.

FIELD

Provided herein are compositions, systems, kits, and methods for treating a disease or
15 condition by administering to a subject an agent that inhibits connexin 46 (Cx46) (e.g.,
ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or
derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine
analog or derivative). In certain embodiments, the agent inhibits connexin 46-mediated gap
junction intercellular connections (GJICs), and/or inhibits connexin 46, but does not inhibit
20 connexin 43, connexin 45, and connexin 37 (e.g., in a human subject). In certain
embodiments, the disease or condition is selected from brain cancer (e.g., glioblastoma),
deafness, cataracts, or a skin disease.

BACKGROUND

25 There is currently an unmet clinical need for more effective treatments for
glioblastoma, which has an incidence of 3 cases per 100,000 people (17). Despite aggressive
therapies including surgery, radiation, and concomitant chemotherapy, these tumors are
uniformly lethal and lead to a median survival of 12-18 months (3, 4). While large-scale
genetic studies have identified key signaling pathway alterations (5), targeted therapies have
30 had minimal success (6-8). Strategies targeting angiogenesis and immune activation that have
demonstrated efficacy in other advanced cancers have generated modest clinical results.

Despite aggressive therapies including surgical resection, radiation, and
chemotherapy, glioblastoma remains uniformly lethal (3, 4). Advances in molecular genetics
have identified key pathway alterations that have driven the development of targeted

therapies (5). However, these approaches have achieved limited clinical success (6-8) due to the development of resistance via pathway redundancy and cellular heterogeneity, including the presence of self-renewing cancer stem cells (CSCs) (9-13). Targeting glioblastoma and the CSC population represents an unmet medical need.

SUMMARY

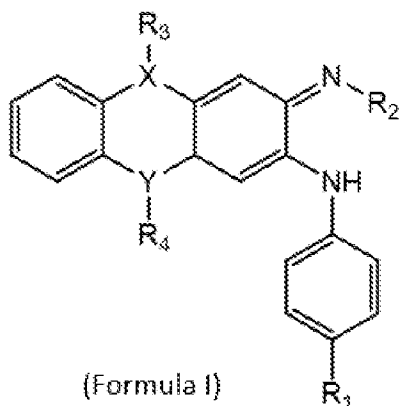
Provided herein are compositions, systems, kits, and methods for treating a disease or condition by administering to a subject an agent that inhibits connexin 46 (Cx46) (e.g., ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine analog or derivative). In certain embodiments, the agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37 (e.g., in a human subject). In certain embodiments, the disease or condition is selected from brain cancer (e.g., glioblastoma), deafness, cataracts, or a skin disease.

In some embodiments, provided herein are methods of treating a disease or condition comprising: administering a composition to a subject with a disease, wherein the composition comprises an agent that: i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; and wherein the disease or condition is selected from glioblastoma, deafness, cataracts, or a skin disease. In particular embodiments, the disease or condition is glioblastoma, and the method further comprises administering at least one of the following: carmustine, lomustine, procarbazine, temozolomide, vincristine, and pembrolizumab.

In some embodiments, provided herein are methods of treating a disease or condition comprising: administering a composition to a subject with a disease, wherein the composition comprises an agent that inhibits connexin 46 (Cx46), wherein said agent comprises a molecule selected from: ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine analog or derivative, wherein the disease or condition is selected from brain cancer (e.g., glioblastoma), deafness, cataracts, or a skin disease. In certain embodiments, the subject is a human.

In certain embodiments, the clofazimine derivative employed in the methods, compositions, systems, and kits herein is as described in U.S. Patent 9,540,336, which is incorporated by reference herein, particularly with respect to the clofazimine derivatives described therein. Such derivatives can be screened for their ability to treat cancer, such as brain cancer (e.g., glioblastoma) using the methods described, for example, in Examples 1, 3, 4, and 5 below.

In some embodiments, the clofazimine analogs employed in the methods, compositions, systems, and kits herein are as described in Formula I below:

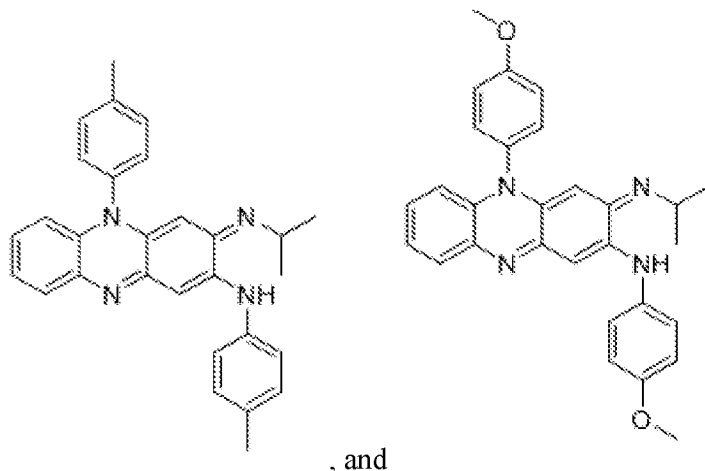


- 10 wherein X is N or S;
 wherein Y is N;
 wherein R₁ is a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;
 wherein R₂ is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted
 15 heteroalkyl of 1-10 backbone atoms in length;
 wherein R₃, when X is N, is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl or substituted heteroalkyl of 1-10 backbone atoms in length, or an aryl or substituted aryl ring;
 wherein R₃, when X is S, is absent, and
 wherein R₄ is absent, or is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or
 20 substituted heteroalkyl of 1-10 backbone atoms in length.

In some embodiments, R₁ is Cl, Br, F, I, CH₃, OCH₃, or -(CH₂)_n-N(CH₃)₂, and n is 0-5. In some embodiments, R₂ is -(CH₂)_n-N(CH₃)₂ and n is 0-5, -CH(CH₃)₂, (CH₂)_n-OCH₃ and n is 0-5, or (CH₂)_n-SCH₃ and n=0-5. In some embodiments, R₃, when X is N, is -(CH₂)_n-N(CH₃)₂ and n is 0-5, -CH(CH₃)₂, (CH₂)_n-OCH₃ and n is 0-5, or (CH₂)_n-SCH₃ and n=0-5. In
 25 some embodiments, R₃, when X is N, is a substituted benzene ring. In some embodiments, R₃, when X is N, is a para-substituted benzene ring. In some embodiments, the benzene ring

- at R₃ is substituted with a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-4 backbone atoms in length. In some embodiments, R₃, when X is S, is absent. In some embodiments, R₄ is absent (e.g., the N attached to the R₄ position is double bonded or there is an H at the R₄ position), or is $-(CH_2)_n-N(CH_3)_2$ and n is 0-5, -
- 5 $CH(CH_3)_2$, $(CH_2)_n-OCH_3$ and n is 0-5, or $(CH_2)_n-SCH_3$ and n=0-5.

In some embodiments, the clofazimine analog comprises a structure selected from:



Clofazimine analogs of Formula I could be screened for their ability to treat cancer, such as brain cancer (e.g., glioblastoma) using the methods described, for example, in

- 10 Examples 1, 3, 4, and 5 below.

In certain embodiments, provided herein are system comprising: a) a composition comprising an agent that: i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; and b) a device configured to deliver the composition to the

15 brain of a subject with glioblastoma. In particular embodiments, at least part of the composition is located inside the device. In certain embodiments, the agent comprises a compound of Formula I. In other embodiments, the agent comprises: i) (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine or ii) (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.

- 20 In particular embodiments, provided herein are composition comprising: a) an agent that: i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; and b) at least one of the following: i) a carrier, wherein the carrier allows the agent to cross the blood-brain barrier of a human subject; and ii) a drug selected from the group consisting of:
- 25 carmustine, lomustine, procarbazine, temozolomide, vincristine, and pembrolizumab.

In certain embodiments, the agent inhibits human connexin 46-mediated GJICs. In further embodiments, the agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and does not inhibit at least one of: connexin 43, connexin 45, and connexin 37. In additional embodiments, the agent does not inhibit all three of: connexin 43, connexin 45, and connexin 37. In some embodiments, the agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and does not inhibit at least one of: human connexin 43, human connexin 45, and human connexin 37. In certain embodiments, the agent does not inhibit all three of: the human connexin 43, the human connexin 45, and the human connexin 37. In certain embodiments, the agent does not alter the vision of the subject. In further embodiments, the subject is a human. In some embodiments, the agent is able to cross the blood brain barrier of the subject.

In particular embodiments, provided herein are systems and kits comprising: a) a composition comprising an agent that inhibits connexin 46 (Cx46), wherein said agent comprises a molecule selected from: ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine analog or derivative, b) a device configured to deliver said composition to the brain of a subject with glioblastoma. In particular embodiments, at least part of said composition is located inside said device. In further embodiments, the device is a Convection Enhanced Delivery (CED) type device or similar device. In certain embodiments, the CED type device is the Cleveland Multiport Catheter, or any of the devices in U.S. Patents 8,808,234, and 8,979,822, both of which are incorporated by reference herein, particularly with respect to the devices described therein. In certain embodiments, the agent comprises a compound of Formula I. In other embodiments, the agent comprises: i) (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine) or ii) (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.

In particular embodiments, provided herein are systems or kits comprising: a) an agent that inhibits connexin 46 (Cx46); and b) a device configured to deliver the composition to a localized area of the subject that has the cancer cells. In some embodiments, the localized area is the brain of the subject, and/or wherein the cancer cells are glioma or glioblastoma cancer cells. In further embodiments, the device comprises a catheter. In additional embodiments, the device comprises a Convection Enhanced Delivery (CED) type device. In further embodiments, at least part of the composition is located inside the device. In additional embodiments, the kits and systems further comprise: c) a chemotherapeutic. In certain embodiments, the agent comprises a compound of Formula I. In other embodiments,

the agent comprises: i) (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine) or ii) (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.

In certain embodiments, the disease or condition is glioblastoma. In other embodiments, the agent is clofazimine or comprises clofazimine. In further embodiments, the agent is ondancetron or comprises ondancetron. In additional embodiments, the agent is clozapine or comprises clozapine. In further embodiments, the agent is testosterone or comprises testosterone. In other embodiments, wherein the disease or condition is glioblastoma, and the method further comprises administering at least one of the following: carmustine, lomustine, procarbazine, temozolomide, vincristine, and pembrolizumab.

DESCRIPTION OF THE FIGURE

Figure 1. Mutational analysis indicates that cell-cell communication is essential to maintain glioblastoma cancer stem cells. (A) Schematic showing the location of Cx46 point mutants in the protein. (B) CSCs from the patient-derived xenograft specimen T4121 were transfected with wildtype or mutant Cx46, and the number of cells was measured on days 0, 1, 3, 7, and 10 after plating using CellTiter-Glo. The values shown are relative to day 0. n = 4 experiments performed in triplicate. * p<0.05, ** p<0.01, *** p<0.001 by two-way ANOVA compared to vector to test for significant differences between the curves. (C-D) Transfected CSCs from the patient-derived xenograft specimens T4121 (C) and T387 (D) were assessed for active caspase 3/7 on day 1 using Caspase-Glo. The values shown are normalized to the CellTiter-Glo signal at day 1, and the values shown are given relative to vector. n = 4 experiments for T4121 and n = 3 for T387, all performed in triplicate. * p<0.05, ** p<0.01, *** p<0.001 by Student's unpaired t-test with Welch's correction compared to vector. (E-F) Transfected CSCs from the patient-derived xenograft specimens T4121 (E) and T387 (F) were plated in a limiting-dilution format (between 1-20 cells/well of a 96-well plate), and the number of spheres per well was counted between days 10 and 14. The stem cell frequency was calculated using the online algorithm described in the Methods section. The values shown are relative to the stem cell frequency of the vector-transfected cells. n = 3 experiments for T4121 and n = 2 for T387, with 24 technical replicates per cell number per experiment. * p<0.05, ** p<0.01, *** p<0.001 by χ^2 test compared to the vector control. Data are represented as mean $\pm$ SEM for B-D and mean $\pm$ range for E-F.

Figure 2. Glioblastoma CSCs express Cx46 mutants. (A-B) CSCs from the patient-derived xenograft specimens T4121 and T387 were transfected with wildtype or mutant Cx46 and lysed for RNA 48 h later. qPCR was performed using Fast Sybr Green, and results were

analyzed using the $\Delta\Delta C_t$ method. Expression is normalized to GAPDH and is shown relative to the vector-transfected cells. $n = 3$ experiments performed in triplicate. All data points are shown, with the mean indicated by a horizontal line. (C) CSCs from the patient-derived xenograft specimen T387 were transfected with wildtype or mutant Cx46, and the number of cells was measured on days 0, 1, 3, 7, and 10 after plating using CellTiter-Glo. The values shown are relative to day 0. $n = 3$ experiments performed in triplicate. ** $p < 0.01$ by two-way ANOVA compared to vector to test for significant differences between the curves. Data are represented as mean $\pm$ SEM.

Figure 3. A screen of FDA-approved small molecules identifies clofazimine as an inhibitor of Cx46-mediated cell-cell communication. (A) Schematic of calcein dye transfer between HeLa cells expressing no exogenous connexin proteins and HeLa cells transfected with Cx43 or Cx46. Cells are labeled with Vybrant DiD (pseudocolored magenta), which cannot pass between cells, and calcein red/orange AM (pseudocolored black), which spreads between cells through gap junctions. (B) Parachute dye transfer assay of parental HeLa cells, stable Cx46-expressing HeLa cells, and transiently transfected Cx43-expressing HeLa cells. Unlabeled cells were plated in a subconfluent monolayer, and dual-labeled cells were added. If gap junctions formed between labeled and unlabeled cells, the calcein dye (shown in black) diffused into cells that were not labeled with DiD (magenta). Scale bar, 50 μm . (C) Schematic of the parachute dye transfer assay with timing used to test the NIH Clinical Collection compounds for inhibition of Cx46-mediated cell-cell communication in stable HeLa-Cx46 cells. A subconfluent monolayer of HeLa-Cx46 cells was plated. A separate population was labeled with calcein red-orange AM and Vybrant DiD and incubated with drugs at 10 μM for 3 h, and dye transfer was imaged after 5 h. (D) Summary graph of the degree to which the drugs from the NIH Clinical Collection inhibited Cx46-mediated GJIC. Percent inhibition is relative to DMSO vehicle control treatment (0%) and the pan-gap junction inhibitor carbenoxolone (100%). (E-F) Validation of the screen results. Labeled cells were treated with increasing concentrations (0.1 μM , 1 μM , 10 μM) of four top hits from the screen and one hit that did not show inhibition (purple). Those cells were then either added to a subconfluent monolayer of unlabeled cells (E) to measure GJIC or plated sparsely (F) to assay dye leakage through hemichannels. Data are normalized to DMSO (0% inhibition) and carbenoxolone (100%), and these experiments were performed in triplicate.

Figure 4. Cx46 is more sensitive than other connexins expressed in GBM to inhibition by clofazimine. (A) Heatmap of connexin mRNA expression in GBM compared to normal brain tissue by both RNA-sequencing and microarray. Data are from TCGA and were

obtained from the GlioVis. Red indicates higher expression compared to normal brain, while blue indicates lower expression than normal brain tissue. (B) Parachute dye transfer assay of HeLa cells expressing different connexin proteins. HeLa cells were transfected with connexin proteins, unlabeled cells were plated in a subconfluent monolayer, and cells dual labeled with Vybrant DiD (shown in magenta) and calcein red/orange AM (shown in black) were treated with DMSO, 1 μ M clofazimine (CFZ), or 200 nM carbenoxolone (CBX) for 3 h and added to the unlabeled cells. The presence of calcein dye (black) in cells that are not magenta indicates GJIC. Scale bar, 50 μ m. (C) Quantification of B. The percent inhibition of GJIC with clofazimine is shown compared to that of vehicle and the pan-gap junction inhibitor carbenoxolone. *** $p < 0.001$ by unpaired Student's t-test with Welch's correction compared to the DMSO-treated control. Data are represented as mean $\pm$ SEM. $n = 3$.

Figure 5. Clofazimine preferentially targets GBM CSCs compared to non-CSCs. (A) Summary of IC₅₀ values for clofazimine in four different patient-derived xenograft matched CSCs and non-CSCs and the NIH3T3 untransformed fibroblast cell line. Cells were treated with increasing concentrations of clofazimine for 3 d, and cell number was measured using CellTiter-Glo. Because non-CSCs never reached a 50% decrease in cell number, an IC₅₀ value could not be calculated. $n =$ at least 3 experiments with cells plated in triplicate. Data are represented as mean $\pm$ SEM. $n = 3$. (B) CSCs from two different patient-derived xenograft specimens were plated into drug-containing medium at increasing cell densities (1, 5, 10, 20 cells/well of a 96-well plate), and the number of wells containing spheres was counted after 10-14 days. The online algorithm described in the Methods section was used to calculate stem cell frequency. *** $p < 0.001$ by χ^2 test compared to the DMSO-treated control. Data are represented as mean $\pm$ range. $n = 3$ experiments, with 24 technical replicates per cell number per experiment. (C) CSCs and non-CSCs from two different patient-derived xenograft specimens were treated with clofazimine for 3 d, and active caspase 3/7 was measured using Caspase-Glo. The values shown are normalized to the number of total cells at the same time point and are relative to the DMSO control for each cell type. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired Student's t-test with Welch's correction compared to the respective DMSO-treated control. Data are represented as mean $\pm$ SEM. $n = 2$ experiments, each performed in triplicate. (D) CSCs were plated in a subconfluent monolayer on Geltrex, treated for 24 h, and microinjected with 2-NBDG (pseudocolored black) and a far-red fluorescently labeled IgG (pseudocolored magenta). Cells were imaged over 2 h, and the number of cells receiving 2-NBDG from each donor cell was quantified. * $p < 0.05$ by unpaired Student's t-test with Welch's correction compared to the DMSO-treated control.

Data are represented as mean $\pm$ SEM. $n = 8$ donors over 7 fields (DMSO) and $n = 4$ donors over two fields (clofazimine). Scale bar, 50 μm . (E) Flow cytometry was used to measure the amount of fluorescent DCF produced from H2CDFDA as a measurement of ROS produced. CSCs were treated concurrently for 24 h with 50 μM temozolomide (TMZ) and for 16 h with 1 μM clofazimine, manually removed from the plate using a cell scraper, and subjected for flow cytometry. Representative data from one of $n = 3$ experiments are shown. (F) Cells were plated in 96-well plates and treated as in E. Active caspase 3/7 was measured using Caspase-Glo. Data are normalized to the total number of cells at that time and are shown relative to the DMSO-treated control. * $p < 0.05$, *** $p < 0.001$ by unpaired Student's t-test with Welch's correction compared to treatment with clofazimine alone. Data are represented as mean $\pm$ SEM. $n = 2$ experiments, each performed in triplicate.

Figure 6. Clofazimine likely acts specifically to inhibit Cx46-mediated GJIC in CSCs. (A) Example IC₅₀ curves for clofazimine in two GBM specimens. Cells were treated with increasing concentrations of clofazimine for 72 h, and cell number was determined using CellTiter Glo. (B) T4121 CSCs were treated with 2 μM clofazimine for 6 hours and subjected to RNA sequencing in triplicate. Volcano plots showing the distribution of changes in transcripts by RNA sequencing. Genes with significant changes are shown as red dots. (C) Heatmap showing the RNA sequencing hits with the largest changes with clofazimine treatment compared to DMSO vehicle. Red indicates higher expression, while blue indicates lower expression within each gene. (D) T4121 CSCs and non-CSCs were lysed for RNA and subjected to qPCR for Kv1.3. Each sample was analyzed in triplicate. * $p < 0.05$ by unpaired Student's t-test with Welch's correction compared to expression in CSCs. Data are represented as mean $\pm$ SEM.

Figure 7. Clofazimine decreases tumor growth in vivo. (A) Female NSG mice were treated with 2.44 mg/kg clofazimine (CFZ) in 200 μl corn oil by intraperitoneal (IP) injection daily for 5 d. On day 8, animals were euthanized, and brains were homogenized in PBS and subjected to mass spectrometry for clofazimine. $n = 3$ brains each for vehicle and clofazimine, and data are shown as mean $\pm$ SEM. The difference is not significant (ns) by unpaired Student's t-test with Welch's correction compared to treatment with vehicle alone (background signal). (B) Female NSG mice were injected with 1×10^6 T4121 CSCs into their right flanks. Three weeks later, when tumors became palpable, animals were treated with 2.44 mg/kg clofazimine in corn oil IP for two weeks. Tumor size was measured using digital calipers, and the change over time is given as the measurement on day 12 relative to day 0. Tumors were excised on day 15, and the final tumor sizes are shown in (C). The scale is

shown in the figure. * $p < 0.05$ by unpaired Student's t-test with Welch's correction compared to treatment with vehicle (corn oil). $n = 10$ mice per arm. All data points as well as the mean and SEM are shown.

Figure 8 show the synthesis scheme for clofazimine analogs 1 and 2 as described in
5 Example 2.

DEFINITIONS

As used herein, the term "heteroalkyl" refers to an alkyl chain (e.g., straight or branched) in which one or more carbons of the alkyl chain are replaced with O, S, or N
10 atoms.

The term "aryl," as used herein, refers to a phenyl group, or a bicyclic or tricyclic aromatic fused ring system. Bicyclic fused ring systems are exemplified by a phenyl group appended to the parent molecular moiety and fused to a phenyl group. Tricyclic fused ring systems are exemplified by a phenyl group appended to the parent molecular moiety and
15 fused to two other phenyl groups. Representative examples of bicyclic aryls include, but are not limited to, naphthyl. Representative examples of tricyclic aryls include, but are not limited to, anthracenyl.

The term "substituent" refers to a group substituted on an atom of the indicated group. When a group or moiety is substituted (e.g., a substituted alkyl), the term "substituted"
20 indicates that one or more (e.g., 1, 2, 3, 4, 5, or 6; in some embodiments 1, 2, or 3; and in other embodiments 1 or 2) hydrogens on the group indicated in the expression using "substituted" are replaced with a selection of recited indicated groups or with a suitable group known to those of skill in the art (e.g., one or more of the groups recited below). Substituent groups include, but are not limited to, halogen, =O, =S, cyano, nitro, fluoroalkyl,
25 alkoxyfluoroalkyl, fluoroalkoxy, alkyl, alkenyl, alkynyl, haloalkyl, haloalkoxy, heteroalkyl, cycloalkyl, cycloalkenyl, aryl, heteroaryl, heterocycle, cycloalkylalkyl, heteroarylalkyl, arylalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkylene, aryloxy, phenoxy, benzyloxy, amino, alkylamino, acylamino, aminoalkyl, arylamino, sulfonylamino, sulfinylamino, sulfonyl, alkylsulfonyl, arylsulfonyl, aminosulfonyl, sulfinyl, -COOH, ketone,
30 amide, carbamate, and acyl.

DETAILED DESCRIPTION

Provided herein are compositions, systems, kits, and methods for treating a disease or condition by administering to a subject an agent that inhibits connexin 46 (Cx46) (e.g.,

ondancetron, an ondancetron analog or derivative, testosterone, a testosterone analog or derivative, clozapine or a clozapine analog or derivative, or clofazimine or a clofazimine analog or derivative). In certain embodiments, the agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or inhibits connexin 46, but does not inhibit
5 connexin 43, connexin 45, and connexin 37 (e.g., in a human subject). In certain embodiments, the disease or condition is selected from brain cancer (e.g., glioblastoma), deafness, cataracts, or a skin disease.

To develop targeting strategies, we interrogated mechanisms that facilitate cellular interactions. Cell-cell communication is the foundation of system complexity and allows
10 groups of cells to expand and respond to perturbations in a coordinated and synchronized manner. Direct cell-cell communication is facilitated by the connexin family of proteins that form gap junctions between adjacent cells. Connexins have been considered tumor suppressors, but we found that glioblastoma CSCs utilize connexins for growth and identified a subunit elevated in CSCs, connexin 46 (Cx46). There are limited connexin-specific
15 inhibitors, and many compounds target connexins in an off-target manner. To identify Cx46-specific inhibitors, we devised an in vitro screening platform to assess direct cell-cell communication and identified several inhibitors of Cx46-mediated communication. In certain embodiments, these hits are used to guide large-scale screening efforts and further develop analogs with high Cx46 specificity and limited off-target effects. In some embodiments,
20 validation occurs in our screening platform and glioblastoma patient-derived models by assessing proliferation, survival, self-renewal, and tumor growth after inhibitor treatment. Cx46 specificity can be tested and compared to other connexins, and off-target effects are assessed using RNA-sequencing.

Tumors are composed of a heterogeneous population of cells containing
25 therapeutically resistant cancer stem cells and non-stem tumor cells. The coordination of complex processes within the tumor requires cells to rapidly modify their phenotypes and is achieved by direct cell-cell communication, which is mediated by gap junctions composed of connexin proteins. Connexins serve three main functions within a cell: 1) mediating passive transfer of small molecules between cells through gap junctions, 2) mediating passive transfer
30 of small molecules between a cell and the extracellular space (i.e., as hemichannels), and 3) acting as a scaffold to mediate protein-protein interactions. Previous reports based on connexin 43 (Cx43) suggested that gap junctions function as tumor suppressors, but this hypothesis fails to take into account differences in connexin-mediated intercellular communication and cargo selectivity. We screened all connexin family members and found

that cancer stem cells express elevated levels of Cx46 compared to non-stem tumor cells. Targeting Cx46 compromised cancer stem cell proliferation, self-renewal, and tumor initiation capacity, demonstrating that connexins can function in a pro-tumorigenic manner and suggesting that targeting Cx46 may be therapeutically relevant for patients with glioblastoma.

Cell-cell communication is a mechanism through which cells establish networks that institute stability via coordinated and synchronized responses. Despite their potential importance during tumorigenesis and in response to therapies, the connexin family of proteins that facilitate direct cell-cell communication has been considered tumor suppressive.

Pharmacological manipulation of connexins remains challenging, as there over 20 family members that are structurally similar. Moreover, compounds that inhibit connexin function do so as a result of off-target effects and lack specificity toward an individual family member. To identify Cx46- specific inhibitors, we developed a cell-cell communication system. This platform identified several compounds that inhibit Cx46- mediated cell communication, and functional studies in glioblastoma patient-derived models confirm that one of the lead compounds reduces cancer stem cell growth. In certain embodiments, the Cx46 inhibitors, and screening methods to identify such inhibitors, are employed to great glioblastoma and other conditions, such as deafness, cataracts, and disorders of the skin.

Based on our interest in targeting connexin 46 (Cx46) in glioblastoma cancer stem cells and the general lack of Cx46-specific inhibitors, we engineered HeLa cells to express Cx46. HeLa cells were chosen due to their low baseline connexin expression and inability to establish functional gap junctions between adjacent cells. Cx46-expressing HeLa cells gained the ability to form functional gap junction subunits, and we used these cells to identify inhibitors that attenuated cell-cell communication. We screened the NIH Clinical Collection and identified several compounds that potently reduce cell-cell communication, including ondansetron, testosterone, clozapine, and clofazimine. We further tested the efficacy of clofazimine and found that it displayed greater inhibition of Cx46 compared to other connexins expressed in the brain and in brain tumors (including Cx43). In our glioblastoma patient-derived models, clofazimine potently inhibited cancer stem cell growth and increased tumor latency. Our analysis suggests that clofazimine crosses the blood-brain barrier, and our molecular modeling studies suggest that clofazimine binds to the extracellular portion of Cx46 (data not shown), making it a good compound, and a good parent compound for future studies proposed in this application. In certain embodiments, this initial screening effort is

employed to identify and develop Cx46 inhibitors for use in glioblastoma, specifically targeting cancer stem cells.

In certain embodiments, analogs or derivatives of ondancetron, testosterone, clozapine, and clofazimine are employed in the methods, compositions, kits, and systems herein (e.g., to treat glioblastoma or other diseases). Examples of clofazimine analogs and derivatives are found in U.S. Patent 9,540,336; Barteselli et al., Bioorg Med Chem. 2015 Jan 1;23(1):55-65; and Lu et al., Antimicrob Agents Chemother. 2011 Nov;55(11):5185-93, all three of which are incorporated herein in their entireties, specifically for the clofazimine analogs and derivatives described therein. In other embodiments, the clofazimine analogs are as described in Formula I herein.

EXAMPLES

EXAMPLE 1

Gap junction-mediated cell-cell communication enables tumor cells to synchronize the execution of complex processes. Despite the connexin family of gap junction proteins being considered tumor suppressors, we previously found that glioblastoma cancer stem cells (CSCs) express higher levels of Cx46 compared to non-stem tumor cells, and this was necessary and sufficient for CSC maintenance. As part of a Cx46 targeting strategy, we utilized point mutants to disrupt specific functions of Cx46 and found that gap junction coupling was an important function of Cx46 for CSCs. Based on this finding, we screened a clinically relevant library of small molecules and identified clofazimine as an inhibitor of Cx46-specific cell-cell communication. Clofazimine attenuated proliferation, self-renewal, and tumor growth and synergized with temozolomide to induce apoptosis. These data indicate that, in some embodiments, clofazimine or other Cx46 inhibitor, without or without other chemotherapies (e.g., standard-of-care therapies) could be used to target glioblastoma CSCs. Furthermore, these results demonstrate the importance of targeting cell-cell communication as an anti-cancer therapy.

Glioblastoma (GBM; grade IV glioma), the most commonly occurring primary malignant brain tumor, remains uniformly fatal despite aggressive therapy including surgery, radiation, and chemotherapy. Research advances have increased the understanding of the disease and improved therapies, but patient prognosis remains poor, with a median survival of only 14-16 months, and 5-year-survival rates remain less than 3% (McGirt et al., 2009; Stupp et al., 2009). One factor underlying the difficulty in treating GBM is the cellular

heterogeneity found within these tumors. One of these cell populations, termed cancer stem cells (CSCs), exhibits essential characteristics of sustained self-renewal, persistent proliferation, and the ability to initiate tumors if transplanted into mice (Lathia et al., 2015) and also displays resistance to the GBM standard-of-care therapies radiation and
5 temozolomide (Bao et al., 2006; Liu et al., 2006).

Cell-cell communication is mediated through the connexin family of proteins and the gap junction (GJ) channels these proteins comprise. Six connexin proteins assemble into a channel through the plasma membrane that can exchange small molecules between the cell and the extracellular space as hemichannels. When these channels dock with a compatible
10 hexamer on a neighboring cell, a GJ is formed. GJ intercellular communication (GJIC) exchanges ions, siRNAs, small metabolites such as glucose, antioxidants, and peptides between cells, allowing them to coordinate their phenotypes and respond to environmental conditions (Goodenough and Paul, 2009). Connexin proteins serve three main cellular functions: exchange of small molecules between cells as gap junctions (Goodenough and
15 Paul, 2009), exchange of small molecules between a cell and the extracellular space as hemichannels (Goodenough and Paul, 2003; Stout et al., 2004), and intracellular protein-protein interactions (Goodenough and Paul, 2009; Leithe et al., 2018).

GBM CSCs express higher levels of Cx46 compared to non-stem tumor cells (non-CSCs), and Cx46 is required for CSC proliferation, survival, self-renewal, and tumor
20 formation (Hitomi et al., 2015). Pan-gap junction inhibitors slowed tumor growth in mice with intracranial tumors, but these compounds inhibit connexins as an off-target effect. For this reason, these compounds would likely cause side effects in patients based on their broad effects targeting multiple connexins that play essential roles in many normal organs. In this Example, we used mutational analysis to identify the essential function of Cx46 in GBM
25 CSCs to be cell-cell communication through gap junctions (GJIC). Therefore, targeting of CSCs through specific inhibition of Cx46 would slow tumor growth and result in therapy for patients with GBM. A screen of FDA-approved small molecules identified the anti-leprosy drug clofazimine as a preferential inhibitor of Cx46-mediated cell-cell communication and GBM CSC maintenance, indicating that this compound could be used for patients with GBM.

30

RESULTS

Cx46-mediated cell-cell communication is essential to maintain glioblastoma cancer stem cells.

5 To develop a strategy to inhibit Cx46, we first sought to determine the function of Cx46 required to maintain GBM CSC properties. To achieve this, we identified a panel of Cx46 mutations that would allow us to deduce the individual importance of GJIC and hemichannel activity. Two Cx46 point mutations have been reported in human patients with cataracts (Hansen et al., 2006; Santhiya et al., 2010). These mutations, L11S and T19M, are
10 both located in the N-terminal tail of the Cx46 protein (Fig. 1A) and have been functionally investigated (Tong et al., 2015; Tong et al., 2013). When co-expressed in *Xenopus* oocytes with wild-type Cx46, the presence of the L11S mutation in the context of rat Cx46 dramatically reduced both GJIC and hemichannel activity (Tong et al., 2013). In contrast, co-expression of the rat Cx46 T19M mutant with wild-type Cx46 increased hemichannel activity
15 but did not affect GJIC of *Xenopus* oocytes (Tong et al., 2015). We also utilized a “cysless” mutant previously engineered in Cx43 that disrupts the three disulfide bonds necessary to maintain the structure of connexins required for gap junction docking. This mutant was reported to completely block GJIC without affecting hemichannel activity of Cx43 in *Xenopus* oocytes and ovarian granulosa cells (Bao et al., 2004; Tong et al., 2007).

20 We introduced these mutations into human Cx46 cDNA and transfected GBM CSCs isolated from two different patient-derived xenografts (T4121 and T387) with the three constructs. Using qPCR, we were able to detect the expression of each Cx46 mutant in our CSCs at the mRNA level (Fig. 2A-B). Expression of the Cx46 T19M mutant or overexpression of wild-type Cx46 had little effect on CSC proliferation, apoptosis, or self-renewal, a hallmark of the CSC state, which was assessed by limiting dilution analysis (Fig. 25 1C-F, Fig. 2C), while we observed slight decreases in proliferation and self-renewal with expression of Cx46 L11S. However, expression of the Cx46 cysless mutant induced a decrease in CSC proliferation, an increase in apoptosis, and a decrease in self-renewal in both patient-derived specimens. This observation demonstrates that the cysless mutant, which has
30 been shown to have the greatest effect on cell-cell communication, also has the greatest effect on CSC maintenance and led us to conclude that GJIC mediated by Cx46 is essential to maintain GBM CSC proliferation, survival, and self-renewal.

A screen of FDA-approved small molecules identifies clofazimine as an inhibitor of Cx46-mediated cell-cell communication.

Based on our observation that GBM CSCs require Cx46-mediated GJIC for survival, we designed an assay system to screen for inhibitors of this process. It is noted that this screen can be used to screen not only FDA-approved small molecules, but other small molecules, such as a small molecule library or particular candidate small molecules.

We assessed GJIC using a quantitative calcein transfer assay (Fig. 3A) (Hitomi et al., 2015), a modification of the parachute dye-uptake assay (Ziambaras et al., 1998). In this assay, cells labeled with both a gap junction-permeable dye (calcein red/orange AM, shown in black) and a non-spreading membrane dye (DiD) were added to a subconfluent monolayer of unlabeled cells. The formation of GJs is indicated by membrane dye-negative cells that become calcein positive with time. HeLa cells express low levels of endogenous connexins (Elfgang et al., 1995) and display minimal dye coupling (Fig. 3B). However, stable expression of Cx46 or transient expression of Cx43 in HeLa cells established functional gap junctions and coupling between cells, as evidenced by the spread of calcein dye (shown in black) between cells (Fig. 3B). Using stable Cx46-expressing HeLa cells, we then screened the 727 compounds of the NIH Clinical Collection of FDA-approved small molecules for their ability to inhibit Cx46-mediated GJIC at a concentration of 10 μ M over a treatment time of 3 hours (Fig. 3C). The spread of calcein between treated cells was imaged after 5 hours and compared to both vehicle (DMSO) treatment and treatment with the pan-gap junction inhibitor carbenoxolone (CBX; 200 nM). We identified a number of compounds that blocked Cx46-mediated GJIC compared to CBX as a positive control (Fig. 3D). A secondary screen of the top hits at concentrations between 0.1 μ M and 10 μ M found that the FDA-approved anti-mycobacterial drug clofazimine inhibited Cx46 GJIC at the lowest concentrations compared to the other hits (Fig. 3E), with little effect on Cx46 hemichannel activity (Fig. 3F). Together, these results demonstrate that clofazimine is a candidate to inhibit Cx46 GJIC without affecting potential hemichannel activity.

Cx46 is more sensitive than other connexins expressed in GBM to inhibition by clofazimine.

An ideal targeting strategy for GBM CSCs expressing Cx46 would preferentially target Cx46 and not the other 20 human connexins. To test the specificity of clofazimine for Cx46, we first screened for the additional connexins expressed in GBM using bioinformatics. Using both RNA-sequencing and microarray data from the Gliovis database (web site at: gliovis.bioinfo.cnio.es/), we identified the connexins most highly expressed in GBM

compared to normal brain (Fig. 34A). In addition to Cx46, which was the most highly expressed relative to normal brain tissue, Cx45 and Cx37 were also detected at higher levels in GBM. We also screened clofazimine against Cx43, the most ubiquitously expressed connexin throughout the body (Oyamada et al., 2005). HeLa cells expressing any of these four connexins displayed GJ coupling, as evidenced by the spread of calcein dye (black) from DiD (shown in magenta)-labeled donor cells to unlabeled recipient cells (Fig. 4B). As expected, the pan-gap junction inhibitor CBX inhibited calcein spread for each connexin. However, while coupling of HeLa cells expressing Cx46 was blocked by clofazimine, cells expressing Cx43, Cx37, and Cx45 continued to exhibit GJIC even in the presence of clofazimine (Fig. 4C). These data indicate that of the connexins tested, clofazimine was specific for inhibition of Cx46-mediated GJIC.

Clofazimine preferentially targets GBM CSCs compared to non-CSCs.

It was thought that clofazimine would specifically target GBM CSCs compared to non-CSCs. Treatment of CSCs and non-CSCs with increasing concentrations of clofazimine from 0.05 μ M to 5 μ M allowed us to calculate IC₅₀ values of approximately 2 μ M for the CSC population of four different patient-derived xenograft specimens (Fig. 5A). In contrast, the non-CSC population never reached 50% growth inhibition within the same concentration range of clofazimine. For comparison, the IC₅₀ of the immortalized, non-transformed fibroblast cell line NIH3T3 was measured at approximately 86 μ M, indicating that CSC growth was dramatically more sensitive than that of other cell types to clofazimine. Limiting dilution analysis showed a significant and striking effect of clofazimine on CSC self-renewal, even at concentrations where proliferation was only minimally affected (0.5 μ M; Fig. 5B and Fig. 4A). This inhibition of CSC growth and self-renewal was accompanied by a concentration-dependent increase in apoptosis in the CSC population, with minimal induction of apoptosis in the non-stem cells (Fig. 5C).

Based on our data that clofazimine inhibited dye coupling in HeLa cells expressing Cx46 and not other connexins (Fig. 4B), we hypothesized that clofazimine was similarly acting through an inhibition of GJIC in CSCs. Indeed, treatment with clofazimine inhibited the spread of the fluorescent glucose analog 2-NBDG microinjected in CSCs compared to vehicle (Fig. 5D), confirming that clofazimine is able to inhibit GJIC in CSCs. To further test whether clofazimine induced additional off-target effects, we performed RNA-sequencing on CSCs from xenograft specimen T4121 treated with 2 μ M clofazimine for a short time period of 6 hours. Increases and decreases in transcript expression with treatment compared to

vehicle were relatively modest, with changes falling within 3-fold of the value of the vehicle-treated samples (Fig. 6B-C). We performed functional gene annotation and pathway enrichment analysis on the top differentially expressed genes (web site at: david.ncicrf.gov/) and found no significant pathway enrichment within reported gene groups with short-term exposure to the drug. As expected, we did not observe any transcriptional off-target effects of clofazimine. Clofazimine has been reported to target GBM cells by affecting the function of the membrane potassium channel Kv1.3, which is highly expressed in many cancer cell lines compared to normal tissue (Leanza et al., 2015; Venturini et al., 2017). We therefore tested our CSCs and non-CSCs to determine whether higher levels of Kv1.3 in the CSCs could be responsible for their sensitivity to clofazimine. However, GBM CSCs from the patient-derived xenograft T4121, which are more sensitive to clofazimine than their non-stem counterparts, expressed approximately 4-fold less Kv1.3 transcript than non-CSCs (Fig. 6D), suggesting that the enhanced sensitivity to clofazimine of CSCs is not due to an effect on Kv1.3 channels.

Inhibition of GJs has been reported to increase the cellular levels of reactive oxygen species (ROS) (Giardina et al., 2007; Le et al., 2014; Zundorf et al., 2007). As expected, treatment with 1 μ M clofazimine for 3 days led to an increase in intracellular ROS as measured by production of fluorescent DCF from H2DCFDA and detected using flow cytometry (Fig. 5E). Based on our observations that clofazimine is toxic to GBM CSCs, we combined clofazimine with temozolomide, GBM standard-of-care chemotherapy. Temozolomide alone (50 μ M) did not increase ROS compared to DMSO vehicle treatment, but a combination of temozolomide with clofazimine further increased ROS above the level observed for clofazimine alone. This increase in ROS was accompanied by a significant increase in apoptosis in cells treated with both temozolomide and clofazimine compared to either compound alone (Fig. 5F). Together, these results indicate that clofazimine inhibits GBM CSC growth, survival, and self-renewal, likely through its effects on Cx46-mediated GJIC, and combines with GBM standard-of-care therapies to further increase tumor cell death.

Clofazimine decreases tumor growth in vivo.

The current World Health Organization (WHO) dosing schedule of clofazimine for multibacillary leprosy includes one monthly dose of 300 mg and an additional 50 mg daily in combination with the drugs dapson and rifampicin for a period of 12 months ((Fischer, 2017). To determine whether clofazimine inhibits tumor growth in vivo, we selected a dosage

equivalent to the maximum recommended daily human dose, 200 mg/day (2.44 mg/kg based on an average body weight of 80 kg), solubilized in corn oil and delivered via intraperitoneal injection (IP). At this dose, we were unable to detect significant accumulation of clofazimine in the brain (Fig. 7A), and we also observed low penetration of the blood-brain barrier by clofazimine in mice. For these reasons, rather than treating mice with intracranial tumors, we instead treated animals bearing flank tumors generated by implantation of CSCs from the PDX specimen T4121. Treatment with 2.44 mg/kg clofazimine by IP for two weeks led to a significant decrease in tumor growth as measured using digital calipers (Fig. 7B), with the final tumor size shown in Fig. 7C. As the normal tissue distribution of Cx46 is primarily in the lens, we also tested whether inhibition of Cx46 had an effect on animal vision and observed no significant changes compared to treatment with vehicle. Together, these results indicate that clofazimine targeting of Cx46-mediated GJIC is able to slow tumor growth without impacting other major Cx46 functions, including vision.

Connexin proteins serve three main cellular functions: exchange of small molecules between cells, exchange of small molecules between a cell and the extracellular space, and intracellular protein-protein interactions. It was previously shown that Cx46 is required for GBM CSC proliferative ability, survival, self-renewal, and tumor formation (Hitomi et al., 2015). Here, using point mutations that disrupt specific functions of the protein, we show that the essential function of Cx46 in these cells is the formation of functional Cx46 GJs. This contrasts with the hypothesis that aberrant hemichannel activity of connexins underlies their role in pathologies (Kim et al., 2016; Leybaert et al., 2017) and suggests that therapies designed to target GJIC mediated by specific connexins may be valuable for certain diseases, including GBM.

To this end, we screened FDA-approved compounds for Cx46 GJIC inhibitors and identified the anti-leprosy drug clofazimine, which inhibited GBM CSC cell-cell communication; decreased CSC growth, survival, and self-renewal; and decreased tumor growth in a subcutaneous tumor model. Although pan-gap junction inhibitors are available clinically and have shown efficacy in models (Hitomi et al., 2015), specific inhibitors for connexin isoforms have yet to be identified or developed. The majority of connexin modulators developed so far, most of which are designed to target Cx43 or multiple connexin isoforms, are peptide mimetics that interrupt a specific binding activity of the molecule – either within the molecule or between molecules – and thus affect protein or channel function (Naus and Giaume, 2016). Although little is known about precisely how these mimetics modulate

connexin activity, they possess varying efficiencies at inhibiting and/or stimulating both GJ and hemichannel activity (Evans et al., 2012; Wang et al., 2013). However, due to the homology among connexin isoforms, many of these mimetics fail to exhibit specificity for a specific connexin. In contrast, we show that the small molecule clofazimine is specific for Cx46 compared to Cx43, Cx45, and Cx37. Few small molecules have been identified to target connexins; those that have been developed increase GJIC in astrocytes or specifically target hemichannels, neither of which are relevant to blocking Cx46-mediated GJIC in GBM CSCs (Naus and Giaume, 2016).

Previous studies described an inhibitory role for clofazimine in GBM cells. Venturini et al. observed significant apoptosis in conventional GBM cell lines treated with clofazimine and attributed this cell death to inhibition of the mitochondrial membrane ion channel Kv1.3 (Venturini et al., 2017). We observed similar cell death of GBM CSCs upon treatment with clofazimine, with little effect on non-CSCs. However, we also detected 4-fold higher levels of Kv1.3 transcript in the clofazimine-resistant non-CSC population, suggesting that clofazimine does not act through Kv1.3 inhibition in our hands. Venturini also observed that clofazimine failed to inhibit growth of intracranial syngeneic mouse gliomas (Venturini et al., 2017), which is supported by our observations that clofazimine at human-relevant doses does not cross the blood-brain barrier. Clofazimine was also previously identified in a screen to inhibit growth of the conventional GBM cell line U87 (Jiang et al., 2014). In contrast, rather than screening for compounds that inhibit GBM cell growth in culture, we identified a CSC essential process, Cx46-mediated GJIC, and screened for inhibitors of this mechanism.

Although clofazimine shows promise for treating GBM, there are several challenges to its therapeutic use. We show that clofazimine exhibits minimal penetration of the blood-brain barrier, and its low solubility and high lipophilicity are also barriers to translation for brain tumors. There has been conflicting evidence for whether clofazimine is able to penetrate the brain; while some studies have reported no detectable levels in the brain (Baik et al., 2013; Holdiness, 1989), a recent study detected a level of 156 ng/mL of clofazimine in the brain of mice treated with 25 mg/kg of the drug (Bajjnath et al., 2015). In contrast, using the equivalent of the maximum tolerated human dose (2.44 mg/kg in mice), we were unable to detect clofazimine in the brain using mass spectrometry or the ability for it to cross the blood-brain barrier. This difference may be due to differences in delivery route, solvent, or concentration. Medicinal chemistry derivatization of clofazimine to optimize solubility and blood-brain barrier penetration will allow a more optimal analog of clofazimine for further

pre-clinical and clinical testing and lead to improved next-generation therapies with reduced side effects for patients with GBM.

Experimental Procedures

5

Origin of cells

Established GBM xenografts T4121, T3691, and T387 were previously reported (Alvarado et al., 2016; Bao et al., 2006; Schonberg et al., 2015) and were obtained. L2 cells were obtained from the University of Florida (Deleyrolle et al., 2011; Siebzehnruhl et al., 10 2013). All human GBM samples were originally established under an IRB-approved protocol that facilitated the generation of xenografts in a de-identified manner from excess tissue taken from consented patients. GBM cells were passaged in immune-deficient NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice (obtained from The Jackson Laboratory, Bar Harbor, ME, USA) and dissociated from established mouse xenografts under Cleveland Clinic- 15 approved protocols. Six-week-old female mice were unilaterally injected subcutaneously in the flank with freshly dissociated human GBM cells, and animals were sacrificed by CO₂ asphyxiation and secondary cervical dislocation when tumor volume exceeded 5% of the animal's body weight. HeLa and NIH3T3 cells were obtained from ATCC.

20 Cell culture

Xenograft tumors were dissociated using papain (Worthington Biochemical Corporation, Lakewood, NJ) and cultured overnight in supplemented neurobasal medium (neurobasal medium (Life Technologies) with 2% B27 (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 2 25 mM L-glutamine, 20 ng/mL EGF (R&D Systems, Minneapolis, MN, USA), and 20 ng/mL FGF-2 (R&D Systems)). T4121, T3691, and T398 xenografts were sorted for CD133⁺ and CD133⁻ populations using the CD133 Magnetic Bead Kit for Hematopoietic Cells (CD133/2; Miltenyi Biotech, San Diego, CA, USA). CD133⁺ cells were maintained in supplemented neurobasal. CD133⁻ cells were maintained in DMEM with 5% FBS and 1% pen/strep. L2 30 cells were maintained in divergent media conditions without sorting.

HeLa and NIH3T3 cells were maintained in DMEM with 10% FBS and 1% pen/strep. The HeLa-Cx46 stable cell line was cultured with the addition of 400 µg/mL G418. All cells were grown in a humidified incubator at 37°C with 5% CO₂.

Plasmids and DNA constructs

pCMV-Cx46 was created by inserting the Cx46 cDNA (catalog# RDC0535, R&D Systems) between the HindIII and XbaI sites of pEGFP-N3, excising the GFP tag. This backbone was used for site-directed mutagenesis to introduce the L11S, T19M, and cysless mutations, using the primers shown in Table 1.

Table 1. Primer sequences used.

Primer Name	Sequence (5' – 3')
Cx46 L11S F	AGCTTTCTGGGAAGACTCTCAGAAAATGCACAGGAGCAC (SEQ ID NO:1)
Cx46 L11S R	GTGCTCCTGTGCATTTTCTGAGAGTCTTCCCAGAAAGCT (SEQ ID NO:2)
Cx46 T19M F	AATGCACAGGAGCACTCCATGGTCATCGGCAAGGTTTGG (SEQ ID NO:3)
Cx46 T19M R	CCAAACCTTGCCGATGACCATGGAGTGCTCCTGTGCATT (SEQ ID NO:4)
Cx46 C54A F	GAGCAGTCAGACTTCACCGCCAACACCCAGCAGCCGGGC (SEQ ID NO:5)
Cx46 C54A R	GCCCCGGCTGCTGGGTGTTGGCGGTGAAGTCTGACTGCTC (SEQ ID NO:6)
Cx46 C61A F	AACACCCAGCAGCCGGGCGCCGAGAACGTCTGCTACGAC (SEQ ID NO:7)
Cx46 C61A R	GTCGTAGCAGACGTTCTCGGCGCCCGGCTGCTGGGTGTT (SEQ ID NO:8)
Cx46 C65A F	CCGGGCGCCGAGAACGTCGCCTACGACAGGGCCTTCCCC (SEQ ID NO:9)
Cx46 C65A R	GGGGAAGGCCCTGTCTAGGCGACGTTCTCGGCGCCCGG (SEQ ID NO:10)
Cx46 C181A F	CTGAAGCCGCTCTACCGCGCCGACCGCTGGCCCTGCCCC (SEQ ID NO:11)
Cx46 C181A R	GGGGCAGGGCCAGCGGTTCGGCGCGGTAGAGCGGCTTCAG (SEQ ID NO:12)
Cx46 C186A F	CGCGCCGACCGCTGGCCCCGCCCAACACGGTGGACGCC (SEQ ID NO:13)
Cx46 C186A R	GGCGTCCACCGTGTGGGGGCGGGCCAGCGGTTCGGCGCG (SEQ ID NO:14)
Cx46 C192A F	GCCCCAACACGGTGGACGCCTTCATCTCCAGGCCACG (SEQ ID NO:15)
Cx46 C192A R	CGTGGGCCTGGAGATGAAGGCGTCCACCGTGTGGGGGC (SEQ ID NO:16)
Kv1.3 F	CAAAACGGGCAATTCCACTG (SEQ ID NO:17)
Kv1.3 R	TGAGCACAGCATGTCACTTG (SEQ ID NO:18)
Cx46 F	TGCACAGGAGCACTCCA (SEQ ID NO:19)
Cx46 R	GCGTGGACACGAAGATGAT (SEQ ID NO:20)

The primers for cysless were designed so that the PCR reactions must be performed sequentially from N-terminus to C-terminus.

pLPCX-Cx43-IRES-GFP was obtained from Addgene (#65433). pcDNA3.1/Hygro(+)-GJC1 (Cx45; cloneID: OHu04829) and pcDNA3.1/Hygro(+)-GJA4 (Cx37; cloneID: OHu33346) were obtained from GenScript.

Transfection and establishment of HeLa-Cx46 stable cell line

For GBM CSC transfections, 1x10⁶ cells were plated per well of a 6-well plate adherently on Geltrex (Thermo Fisher Scientific) to obtain a confluence of approximately 75-80%. Six hours later, cells were transfected with Cx46 or its mutant forms using FuGENE HD (Promega) according to the manufacturer's protocol. Briefly, cells were transfected with 5 µg total DNA (4 µg of connexin and 1 µg pEGFP-N3 to track transfection efficiency) using 15 µl FuGENE per well. The following day, cells were removed from the plate using Accutase (BioLegend) and plated for downstream assays. pEGFP-N3 was used as a vector control.

HeLa cells were seeded at 400,000 cells in a 6-well plate and transfected using XtremeGene HP (Roche) according to the manufacturer's protocol. In brief, each well received 2 µg of DNA and 6 µl of XtremeGene reagent. Dye-transfer recipients were plated 24 hours after transfection, and donors were plated and images taken at 48 hours post-transfection. Stable HeLa-Cx46 cells were derived by transfecting HeLa cells with pCMV-Cx46 (without the GFP tag). Cells were selected with G418 (400 µg/mL), and single-cell clones were tested for the ability to exhibit dye coupling.

Compounds

Clofazimine was obtained from Sigma-Aldrich (catalog # C8895) and solubilized in DMSO at a concentration of 10 mM for in vitro experiments and at 0.489 mg/mL in corn oil for in vivo experiments.

Proliferation and apoptosis

For proliferation, IC₅₀, and apoptosis assays, 2,000 cells were plated per well of a white-walled 96-well plate in triplicate. The number of cells was measured using CellTiter Glo (Promega) on days 0, 1, 3, 7, and 10 according to the manufacturer's protocol using ATP content as a surrogate of cell number, and apoptosis was measured using CaspaseGlo 3/7 (Promega) on days 1 and 3 according to the manufacturer's protocol. For the proliferation of GBM CSCs in the presence of Cx46 and Cx46 mutants, similar results were obtained using the DNA-based CyQUANT Direct Cell Proliferation Assay Kit (Thermo Fisher Scientific). For drug treatments, cells were seeded in triplicate at 2,000 cells per well of a 96-well plate, and the appropriate concentration of drug was added 6-24 hours later. Cells were analyzed both at 0 and 72 h after treatment with drug.

Limiting dilution analysis

CSCs were dissociated using Accutase and plated in a 96-well plate at increasing cell numbers (1, 5, 10, and 20 cells/well) with 24 replicates per cell number. Cells were plated into drug-containing media, and the number of wells containing spheres was counted after 10-14 days. An online algorithm (web site: bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009) was used to calculate stem cell frequency. This was repeated at least two times per drug concentration.

cDNA and qPCR

For qPCR, RNA was extracted from cells using TriZOL (Life Technologies) according to the manufacturer's protocol. A total of 1 µg of RNA was used for reverse transcription using a qScript cDNA Synthesis Kit (QuantaBio) according to the manufacturer's recommendations. Equal volumes of cDNA were amplified using Fast SYBR® Green Master Mix (Applied Biosystems) on a Step-One Plus Real-Time PCR system (Applied Biosystems). Data were analyzed using the $\Delta\Delta C_t$ method to calculate relative levels of product. qPCR primers are provided in Table 1.

Screen for Cx46 inhibitors in the NIH Clinical Collection

Non-labeled Cx46-HeLa cells were seeded at 20,000 cells per well in a 96-well plate in DMEM with 10% FBS and 1% pen/strep. The following morning, drugs were added to a concentration of 10 µM to 80 of the wells, leaving 16 for positive and negative inhibition controls. Carbenoxelone (200 nM) was used a positive control for dye transfer inhibition, while negative control wells were left untreated. Separately, a population of calcein red-orange AM/Vybrant DiD dual-labeled Cx46-HeLa cells was generated. These cells were incubated in serum-free DMEM containing calcein red-orange (resuspended in 50 µL of DMSO and used at 1:1000; Thermo Fisher Scientific) and Vybrant DiD (1:500, Thermo Fisher Scientific) at 37°C for 1 h. Following a 3 h incubation of the unlabeled recipients with drug, the dual-labeled donor population was added at a concentration of 3,000 cells/well. These cells were incubated together at 37°C for 5 h and then imaged after 5 hours. Each plate contained 80 drugs and 16 controls, accounting for 9 experimental runs. Each drug was screened one time per drug as a cursory screen. Following the identification of possible targets, a secondary screen of a selection of top hits was performed at drug concentrations of 10 µM, 1 µM, and 0.1 µM.

For screen quantification, calcein fluorescence (red) was used to create a mask to eliminate any cells left entirely unlabeled and any background fluorescence. The Vybrant DiD fluorescence (far red) image was used to create another binary mask to define DiD-positive donor cells. These mask images were given values of 0 (no dye present) or 1 (dye present) and then multiplied by the calcein image. ImageJ particle analysis of the resulting product images provided us with the raw integrated density (RID) of the total calcein dye per imaged cell. The sum of the particle analysis of the product of the calcein mask and the calcein image gave the total calcein amount, and that of the product of the DiD mask and the calcein image gave the amount of calcein retained in the donor cells. Percent transfer was calculated by $(\text{total calcein} - \text{retained calcein}) * 100 / \text{total calcein}$.

For hemichannel function assessment, labeled populations were generated as described above and seeded at 3,000 cells per well. Cells were given an hour to adhere and then imaged every 15 minutes for 5 hours. Loss of calcein through hemichannels was quantified as the percent of dye that was lost at after 5 h compared to time 0.

For HeLa cells expressing different connexin proteins, cells were prepared and imaged as stated above. Images were quantified as the number of unlabeled cells (recipients) receiving calcein dye per donor cell.

For microinjection of CSCs, subconfluent monolayers of cells plated on Geltrex-coated glass coverslips in 35 mm dishes were pretreated for 16 h with the indicated concentration of clofazimine in growth media. Cells were then injected with far-red fluorescent IgG and the fluorescent glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) as described (Hitomi et al., 2015) and imaged as above. Images were again quantified as the number of unlabeled cells (recipients) receiving calcein dye per donor cell.

GlioVis analysis of connexins in GBM

The Cancer Genome Atlas (TCGA) dataset was interrogated using GlioVis (gliovis.bioinfo.cnio.es, citation) for microarray (Agilent-4502A) and RNAseq levels of all available connexin genes. Relative levels of non-tumor and GBM tissues were analyzed, and the fold change is represented as a heat map.

RNA sequencing

T4121 CSCs were treated with clofazimine at 2 μ M for 6 hours and lysed for RNA using a Macherey-Nagel Nucleospin RNA isolation kit. RNA-seq libraries were prepared

using ~10,000 ng of total RNA. Briefly, the protocol included PolyA⁺ RNA selection, cDNA synthesis, end repair, A-base addition, and ligation of the Illumina-indexed adapters according to previously published methods (Zhang et al., 2012). Total transcriptome libraries were prepared as previously described. Library quality and quantity were measured on an
5 Agilent 2100 Bioanalyzer for product size and concentration. Libraries were also precisely quantified by using a KAPA Library Quantification kit prior to loading on the sequencer and pooled at equimolar quantities between samples. Single-end libraries were sequenced with the Illumina HiSeq 2500 (1x5 read length), with sequence coverage up to 20 M total reads.

Single-end transcriptome sequencing reads were aligned to the human reference
10 genome (GRCh37/hg19) using the spliced read mapper TopHat2 (TopHat 2.0.4) (Kim et al., 2013). Gene expression, as fragments per kilobase of exon per million fragments mapped (FPKM; normalized measure of gene expression), was calculated using Cufflinks (Trapnell et al., 2012). We considered differential expression of the gene when the calculated $p < 0.01$ and there was a 1.5-fold difference (increase or decrease).

15 The database for annotation, visualization and integrated discovery (DAVID) analysis was used for functional clustering and annotation of differentially expressed genes (web site: david.abcc.ncifcrf.gov/) (Jiao et al., 2012). DAVID is a web-based online bioinformatics resource that aims to provide tools for pathway mining and the subsequent functional interpretation of large lists of genes/proteins using a comprehensive and exhaustive set of
20 knowledge-base libraries. The publication on the DAVID webserver suggests investigating clusters with an enrichment score ≥ 1.3 , while our highest enrichment score was 1.06, suggesting no major disturbance of any functional pathway/gene ontology group.

Reactive oxygen species (ROS)

25 To measure intracellular ROS, CSCs were treated with 50 μ M temozolomide for 24 h and 1 μ M clofazimine for 16 h. Cells were then collected and incubated with 1 μ M H2DCFDA (Life Technologies) for 15 min at 37°C. Cells were then washed twice in PBS, and the green fluorescent DCF produced was analyzed on a BDFortessa flow cytometer. DAPI exclusion was used to gate for live cells, and H2O2 was used as a positive control for
30 ROS production.

Orthotopic tumors

Six- to eight-week-old immunocompromised female NSG mice were injected with 1×10^6 CSCs from the patient-derived xenograft T4121 into their right flank. Three weeks later, when tumors were palpable, mice were treated IP with clofazimine at 2.44 mg/kg in

corn oil or vehicle for two weeks on weekdays. Tumor width was measured using digital calipers on days 1 and 12 and is provided as the relative change over that time. Animals were sacrificed on day 15, and tumors were excised and imaged. All animal experiments were performed under Cleveland Clinic-approved Institutional Animal Care and Use Committee-
5 approved protocols.

EXAMPLE 2

Synthesis of Clofazimine Analogs 1 and 2

10 This Example describes the synthesis protocol for clofazimine Analogs 1 and 2.

Material for the chemical synthesis: The starting materials, reagents and solvents for the synthesis were procured from the commercial sources like Sigma Aldrich, Fisher Scientific Acros, Oakwood Product, combi-block or chem-Impex and used as such without any further
15 purification. Compounds were purified by either column chromatography using SilicaFlash 63 (200 u, 60 A) under positive house nitrogen pressure or Buchi Flash system using 40 psi pressure and two solvent gradient system.

Synthesis of Phenazine Derivatives: **Scheme 1** (shown in Figure 8) illustrate the overall
20 synthetic route for the phenazine compounds. From the previously reported method using reagents KF/K₂CO₃ with aniline derivatives (**1-2**) with 2-fluoronitrobenzne (**A**) were reacted to yield the secondary amine derivatives (**3-4**). The nitro group was reduced by the 10% Pd/C catalyst under hydrogen atmosphere (40Psi) to produce the diamines (**5-6**). The FeCl₃/HCl aqueous oxidation was performed on the diamine to give phenazine slats (**7-8**) as precipitate.
25 The reaction of isopropylamine with phenazine slats (**7-8**) yield the corresponding phenazine derivatives as clofazamine analogues (**9-10**), as final analogs 1 and 2.

General Procedure for Nitro derivatives: K₂CO₃ (12.8 mmol, 1.0 equiv.) and KF (12.8 1.0 equiv.) were weighed in 200 mL RB in 50 mL dry DMF. Then aniline derivatives (**1-2**, 15.3
30 mmole, 1.2 equivalents), followed by fluoronitrobenzene 2 (12.8 mmole, 1.0 equiv.) were added and the reaction mixture was heated at 130 °C for 16 hours and monitored by TLC at regular intervals. The solid mixture was extracted with Ethyl acetate (4 × 20 mL), washed with 1N HCl (2 × 30 mL), water (2 × 50 mL), dried (Na₂SO₄) and the solvent was removed

under reduced pressure to yield the crude product. It was purified using flash chromatography.

5 *2-Nitro-N-(p-tolyl)aniline (3)*: Flash column were done and Yellow crystalline compound eluted in 3-5 % EtOAc:Hexane, Yield (3.0 g, 56%). ¹H NMR (500 MHz, Chloroform-*d*) δ 9.45 (s, 1H), 8.20 (dd, *J* = 8.6, 1.8 Hz, 1H), 8.08 (dt, *J* = 9.7, 4.8 Hz, 1H), 7.65 (dtt, *J* = 6.1, 4.4, 2.6 Hz, 1H), 7.32 (tdd, *J* = 10.9, 7.7, 2.5 Hz, 3H), 7.22 (d, *J* = 8.1 Hz, 2H), 7.15 (dd, *J* = 11.0, 8.1 Hz, 3H), 6.73 (ddd, *J* = 8.6, 6.9, 1.6 Hz, 1H), 2.38 (s, 3H).

10 *N-(4-Methoxyphenyl)-2-nitroaniline (4)*: Flash column has been done to elute the red yellow crystalline compound in 10% EtOAc:Hexane, Yield 3.4 g (85%). ¹H NMR (500 MHz, Chloroform-*d*) δ 9.40 (s, 1H), 8.20 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.32 (ddd, *J* = 8.7, 6.9, 1.8 Hz, 1H), 7.20 (dd, *J* = 9.0, 2.5 Hz, 2H), 7.02 – 6.98 (m, 1H), 6.98 – 6.92 (m, 2H), 6.71 (ddd, *J* = 8.6, 6.9, 1.5 Hz, 1H), 3.85 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 158.08, 144.65, 135.87,
15 132.63, 131.35, 127.28, 126.76, 116.91, 115.90, 115.12, 55.69.

General procedure for the Nitro reduction to amine:

A 250-mL pressure flask was flushed with nitrogen and then charged with the nitro compound (**3-4**), Ethanol (60 mL) and 10% Pd/C (0.1 equiv.). It was flushed again with
20 nitrogen and sealed with rubber septum. The evacuated flask was filled with hydrogen (40Psi) and it was shaken on a mechanical shaker at 20°C in a hydrogen atmosphere for 2 hours. TLC monitored for the completion of reaction. The solution was filtered over the celite under vacuum. The solvent was removed under reduced pressure to yield the compound **5** and **6**.

25 *N1-(p-Tolyl)benzene-1,2-diamine (5)*: Dark brown red compound was obtained as pure and used such as without further purification Yield 80%. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.09 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.02 (d, *J* = 8.0 Hz, 2H), 6.98 (td, *J* = 7.6, 1.5 Hz, 1H), 6.79 (dd, *J* = 7.9, 1.4 Hz, 1H), 6.77 – 6.73 (m, 1H), 6.70 – 6.67 (m, 2H), 2.27 (s, 3H). ¹³C NMR
30 (126 MHz, CDCl₃) δ 142.65, 141.33, 129.79, 129.43, 128.89, 125.09, 123.86, 119.19, 116.13, 115.80, 20.51.

N1-(4-Methoxyphenyl)benzene-1,2-diamine (6): Dark red color solid, used as such for the next step without any purification, Yield 90%. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.02

(dd, $J = 7.8, 1.8$ Hz, 1H), 6.97 – 6.92 (m, 1H), 6.83 – 6.79 (m, 3H), 6.79 – 6.75 (m, 3H), 6.73 (dd, $J = 7.6, 1.8$ Hz, 1H), 5.10 – 4.90 (m, 1H), 3.76 (d, $J = 2.4$ Hz, 3H), 3.70 (s, 1H).

General Procedure for Phenazine slats: To a solution of the diamino compound (1 Equiv.) in glacial acetic acid (4.5 mL) was added a aqueous solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (8.7 mmole, 3 equiv.) in water (15 mL) and 12 N HCl (0.4 mL, 4.5 equiv.). The reaction was left stirring at room temperature overnight after dilution with 7 mL of H_2O . The reaction was further diluted with 10 mL of H_2O . After 1h, the precipitate was filtered under vacuum, washed with excess water, dried under vacuum and then inside a vacuum desiccator.

3-Imino-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine hydrochloride (7): Dark red/Black color precipitate obtained and used for the next step with any further purification. ^1H NMR (500 MHz, Chloroform- d) δ 7.73 (dd, $J = 7.7, 1.5$ Hz, 1H), 7.50 (d, $J = 7.8$ Hz, 2H), 7.34 (d, $J = 7.9$ Hz, 1H), 7.23 – 7.13 (m, 6H), 7.06 (d, $J = 8.2$ Hz, 1H), 6.96 (s, 1H), 6.92 (s, 1H), 6.69 (d, $J = 8.2$ Hz, 1H), 6.58 – 6.51 (m, 2H), 5.30 (s, 1H), 5.23 (s, 1H), 2.53 (s, 3H), 2.35 (s, 3H).

3-Imino-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine hydrochloride (8): Dark red/Black precipitate was obtained and washed with many time with water dried over the vacuum. The precipitate was dissolved in Ethyl acetate and washed with brine 2-3 times (50 mL) each. Yield 30 %. ^1H NMR (500 MHz, Chloroform- d) δ 8.15 (s, 1H), 7.70 (dd, $J = 7.6, 1.9$ Hz, 1H), 7.32 – 7.28 (m, 2H), 7.21 (qd, $J = 9.3, 4.8$ Hz, 4H), 7.17 – 7.13 (m, 1H), 6.95 – 6.90 (m, 2H), 6.75 (s, 1H), 6.58 – 6.53 (m, 1H), 5.23 (s, 1H), 3.95 (s, 3H), 3.83 (s, 3H).

General procedure for Phenazine isopropyl derivatives: The phenazine hydrochloride (0.1g) was suspended in dioxane or EtOH (1 mL). To this suspension was added isopropylamine (0.2 mL) and the mixture was Heated at 110°C in a sealed pressure tube for 15 h. The solution was filtered and the flask was washed with EtOH (1 mL). The filtrate was diluted with H_2O until it becomes slightly turbid. The compound was crystallized and then recrystallized from aqueous EtOH or purified by the flash chromatography.

(Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine (9): Dark black amorphous solid pure enough for the biological assay. ^1H NMR (500 MHz, Chloroform- d) δ 7.69 – 7.65 (m, 1H), 7.49 (d, $J = 7.6$ Hz, 3H), 7.40 (d, $J = 8.0$ Hz, 3H), 7.21 – 7.16 (m, 8H), 7.16 – 7.09 (m, 7H), 6.95 (d, $J = 7.9$ Hz, 2H), 6.76 (t, $J = 7.5$ Hz, 2H), 6.62 (d, $J = 5.2$ Hz,

2H), 6.48 (d, $J = 8.2$ Hz, 2H), 6.09 (d, $J = 4.5$ Hz, 2H), 5.96 (d, $J = 8.1$ Hz, 2H) 3.43 (q, $J = 6.4$ Hz, 2H), 2.44 (s, 3H), 2.40 (s, 3H), 1.07 (d, $J = 6.3$ Hz, 6H). HRMS Exact mass: Chemical Formula: C₂₉H₂₈N₄ Predicted value 433.2387 and obtained value 433.2387 for the [M + H]⁺

5

(*Z*)-3-(Isopropylimino)-*N*,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine (**10**): Dark red/Black color amorphous solid, pure enough for the biological assay, yield 40%. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.65 (d, $J = 7.8$ Hz, 1H), 7.24 – 7.15 (m, 5H), 7.15 – 7.04 (m, 3H), 6.91 (d, $J = 8.4$ Hz, 2H), 6.74 (d, $J = 4.3$ Hz, 1H), 6.67 (s, 1H), 6.50 (d, $J = 8.7$ Hz, 2H), 5.33 (s, 1H), 3.95 (d, $J = 4.6$ Hz, 3H), 3.83 (d, $J = 2.5$ Hz, 3H), 3.51 – 3.43 (m, 1H), 1.08 (d, $J = 6.2$ Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 159.99, 156.27, 150.84, 145.45, 135.77, 135.27, 132.88, 132.00, 130.28, 129.90, 129.81, 129.62, 128.14, 127.79, 127.43, 126.85, 124.42, 122.75, 122.46, 122.39, 116.30, 116.25, 116.01, 114.67, 114.55, 114.36, 114.13, 113.96, 97.60, 89.01, 55.66, 55.64, 55.56, 49.24, 23.61. HRMS: Chemical Formula: C₂₉H₂₈N₄O₂ Predicted value 465.2285 and obtained value 465.2290 for the [M + H]⁺

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EXAMPLE 3

Testing Clofazimine Analogs In Vitro

This Example describes how one could test clofazimine Analog 1 ((*Z*)-3-(Isopropylimino)-*N*,5-di-*p*-tolyl-3,5-dihydrophenazin-2-amine), clofazimine Analog 2 ((*Z*)-3-(Isopropylimino)-*N*,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine), and a candidate clofazimine analog (e.g., an analog encompassed by Formula I herein) to treat cancer cells in vitro.

20

To obtain IC₅₀ values, CSCs (e.g., glioblastoma CSCs) and non-CSCs (e.g., glioblastoma non-CSCs) are treated with increasing concentrations of Analog 1, Analog 2, and/or a candidate clofazimine analog, and proliferation is measured using CellTiter Glo (Promega) and trypan blue-based cell counting. Once a concentration range has been established that impacts CSCs, cell survival and self-renewal is assessed. Cell survival is measured based on caspase 3/7 activity (Promega) and annexin V/PI positivity. Self-renewal is assessed via sphere formation using in vitro limiting dilution analysis as per previous reports based the algorithm in Hu et al., Journal of immunological methods. 2009;347:70-78, herein incorporated by reference in its entirety.

25

30

To assess the impact of clofazimine analogs, and candidate clofazimine analogs, on stem cell signaling, treated cells are assessed for changes in stem cell signaling nodes

(phospho-STAT3 (tyrosine 705, serine 727), phospho-Akt (serine 473), SOX2, NANOG) after treatment via immunoblot. In parallel, preliminary RNA-sequencing is performed on treated cells and compared to a larger set of stem cell genes via gene set enrichment analysis, using one sample per molecular subtype group in triplicate. Gap junction mediated cell-cell communication are assessed via time-lapse imaging of microinjected cells in the presence of clofazimine analogs and candidate analogs.

EXAMPLE 4

Testing Analogs 1 and 2 In Vitro

This Example describes the testing of clofazimine (CLFZ), clofazimine Analog 1 ((Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine) and clofazimine Analog 2 ((Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine) to treat glioblastoma cancer cells in vitro. This in vitro testing was carried out in a manner similar to Example 1 and Example 3 using xenograft-derived cell lines T4121, T387, and L0, which are cancer stem cell (CSC) glioblastoma cells lines. Results are shown in Table 2 below. Table 2 shows that clofazimine (CLFZ), Analog 1, and Analog 2, all had similar IC₅₀ values in multiple PDX models (T4121, T387, L0).

TABLE 2

Patient-derived xenograft model	IC ₅₀ (μM)		
	CLFZ	Analog 1	Analog 2
T4121	0.59	1.27	1.54
T387	1.08	1.24	1.27
L0	0.54	1.08	1.16

EXAMPLE 5

Testing Clofazimine Analogs In Vivo

This Example describes how one could test Analog 1 ((Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine), Analog 2 ((Z)-3-(Isopropylimino)-N,5-bis(4-

methoxyphenyl)-3,5-dihydrophenazin-2-amine), and a candidate clofazimine analog (e.g., an analog encompassed by Formula I herein) to treat cancer cells (e.g., glioblastoma) in vivo.

To test efficacy of Analogs 1 and 2, or a candidate clofazimine analog in vivo, approximately 100 patient-derived CSCs from one classical, mesenchymal, proneural model are injected intracranially into immune-compromised NSG (NOD.Cg-Prkdcscid
5 *Il2rgtm1Wjl/SzJ*) mice. Treatment begins 3 days after implantation, and the compounds are administered via gavage or intraperitoneal injection. Time until endpoint (onset of neurological symptoms) is monitored for both inhibitor- and vehicle-treated mice. After saline perfusion to eliminate intravascular drug concentration, mass spectrometry is used to
10 measure overall brain penetration and histology is used to determine changes in proliferation (phospho-histone H3, Ki-67), apoptosis (cleaved caspase 3, TUNEL assay), and CSCs (SOX2, phospho-STAT3 (serine 727)). All studies are compared to vehicle treated groups.

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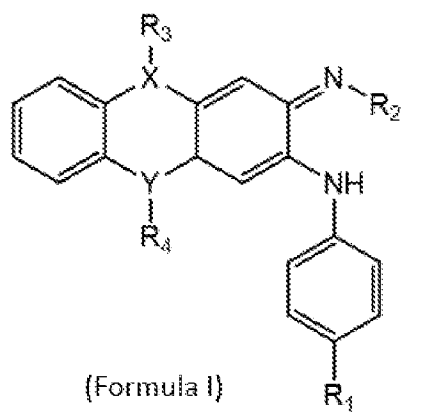
25 All publications and patents mentioned in the specification and/or listed below are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope described herein.

CLAIMS

We Claim:

1. A method of treating a disease or condition comprising: administering a composition to a subject with a disease,
wherein said composition comprises an agent that:
 - i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or
 - ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; andwherein said disease or condition is selected from glioblastoma, deafness, cataracts, or a skin disease.
2. The method of Claim 1, wherein said agent inhibits human connexin 46-mediated GJICs.
3. The method of Claim 1, wherein said agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and does not inhibit at least one of: connexin 43, connexin 45, and connexin 37.
4. The method of Claim 3, wherein said agent does not inhibit all three of: connexin 43, connexin 45, and connexin 37.
5. The method of Claim 1, wherein said agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and does not inhibit at least one of: human connexin 43, human connexin 45, and human connexin 37.
6. The method of Claim 5, wherein said agent does not inhibit all three of: said human connexin 43, said human connexin 45, and said human connexin 37.
7. The method of Claim 1, wherein said agent does not alter the vision of said subject.
8. The method of Claim 1, wherein said subject is a human, and wherein said agent is able to cross the blood brain barrier of said subject.

9. The method of Claim 1, wherein said disease or condition is glioblastoma.
10. The method of Claim 1, wherein said agent is clofazimine.
11. The method of Claim 1, wherein said agent comprises clofazimine.
12. The method of Claim 1, wherein said agent comprises: i) (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine) or ii) (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.
13. The method of Claim 1, wherein said agent comprises a compound of Formula I:



wherein X is N or S;

wherein Y is N;

wherein R₁ is a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

wherein R₂ is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

wherein R₃, when X is N, is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl or substituted heteroalkyl of 1-10 backbone atoms in length, or an aryl or substituted aryl ring;

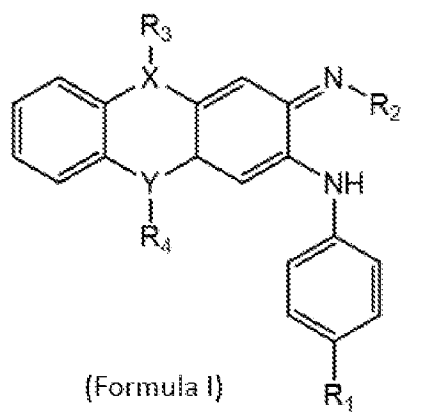
wherein R₃, when X is S, is absent, and

wherein R₄ is absent, or is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length.

14. The method of Claim 13, wherein X is N.

15. The method of Claim 1, wherein said disease or condition is glioblastoma, and the method further comprises administering at least one of the following: carmustine, lomustine, procarbazine, temozolomide, vincristine, and pembrolizumab.
16. The method of Claim 1, wherein said disease is cancer, and wherein said administering comprises administering said agent locally to said subject in an area of said cancer cells.
17. The method of Claim 16, wherein said agent is delivered with a catheter.
18. The method of Claim 16, wherein said agent is delivered with a Convection Enhanced Delivery (CED) type device to said brain of said subject.
19. A system or kit comprising:
- a) a composition comprising an agent that:
 - i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or
 - ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; and
 - b) a device configured to deliver said composition to the brain of a subject with glioblastoma.
20. The system or kit of Claim 19, wherein said agent inhibits human connexin 46-mediated GJICs.
21. The system or kit of Claim 19, wherein said agent inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and does not inhibit at least one of: connexin 43, connexin 45, and connexin 37.
22. The system or kit of Claim 19, wherein said agent does not inhibit all three of: connexin 43, connexin 45, and connexin 37.

23. The system or kit of Claim 19, wherein at least part of said composition is located inside said device.
24. The system or kit of Claim 19, wherein said device comprises a Convection Enhanced Delivery (CED) type device.
25. The system or kit of Claim 19, wherein said agent comprises: i) (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine) or ii) (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.
26. The system or kit of Claim 19, wherein said agent comprises a compound of Formula I:



wherein X is N or S;

wherein Y is N;

wherein R₁ is a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

wherein R₂ is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

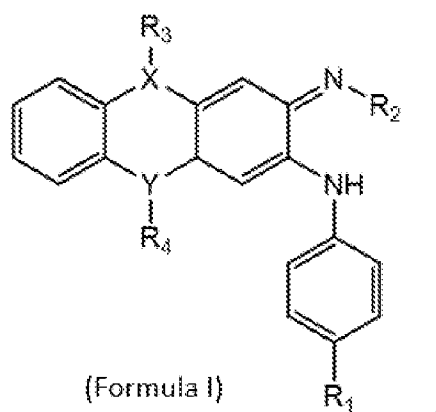
wherein R₃, when X is N, is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl or substituted heteroalkyl of 1-10 backbone atoms in length, or an aryl or substituted aryl ring;

wherein R₃, when X is S, is absent, and

wherein R₄ is absent, or is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length.

27. A composition comprising:
- a) an agent that:
 - i) inhibits connexin 46-mediated gap junction intercellular connections (GJICs), and/or
 - ii) inhibits connexin 46, but does not inhibit connexin 43, connexin 45, and connexin 37; and
 - b) at least one of the following:
 - i) a carrier, wherein said carrier allows said agent to cross the blood-brain barrier of a human subject; and
 - ii) a drug selected from the group consisting of: carmustine, lomustine, procarbazine, temozolomide, vincristine, and pembrolizumab.

28. A composition comprising a compound of Formula I:



wherein X is N or S;

wherein Y is N;

wherein R₁ is a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

wherein R₂ is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length;

wherein R₃, when X is N, is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl or substituted heteroalkyl of 1-10 backbone atoms in length, or an aryl or substituted aryl ring;

wherein R₃, when X is S, is absent, and

wherein R₄ is absent, or is an alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-10 backbone atoms in length.

29. The composition of Claim 28, wherein R_1 is Cl, Br, F, I, CH_3 , OCH_3 , or $-(CH_2)_n-N(CH_3)_2$, and n is 0-5.
30. The composition of Claim 28, wherein R_2 is $-(CH_2)_n-N(CH_3)_2$ and n is 0-5, $-CH(CH_3)_2$, $(CH_2)_n-OCH_3$ and n is 0-5, or $(CH_2)_n-SCH_3$ and $n=0-5$.
31. The composition of Claim 28, wherein R_3 , when X is N, is $-(CH_2)_n-N(CH_3)_2$ and n is 0-5, $-CH(CH_3)_2$, $(CH_2)_n-OCH_3$ and n is 0-5, or $(CH_2)_n-SCH_3$ and $n=0-5$.
32. The composition of Claim 28, wherein R_3 , when X is N, is a substituted benzene ring.
33. The composition of Claim 28, wherein R_3 , when X is N, is a para-substituted benzene ring.
34. The composition of Claim 33, wherein the benzene ring at R_3 is substituted with a halogen, alkyl, heteroalkyl, substituted alkyl, heteroalkyl, or substituted heteroalkyl of 1-4 backbone atoms in length.
35. The composition of Claim 28, wherein R_3 , when X is S, is absent.
36. The composition of Claim 28, wherein R_4 is absent, or is $-(CH_2)_n-N(CH_3)_2$ and n is 0-5, $-CH(CH_3)_2$, $(CH_2)_n-OCH_3$ and n is 0-5, or $(CH_2)_n-SCH_3$ and $n=0-5$.
37. The composition of Claim 28, wherein said compound comprises (Z)-3-(Isopropylimino)-N,5-di-p-tolyl-3,5-dihydrophenazin-2-amine.
38. The composition of Claim 28, wherein said compound comprises (Z)-3-(Isopropylimino)-N,5-bis(4-methoxyphenyl)-3,5-dihydrophenazin-2-amine.

FIG. 1

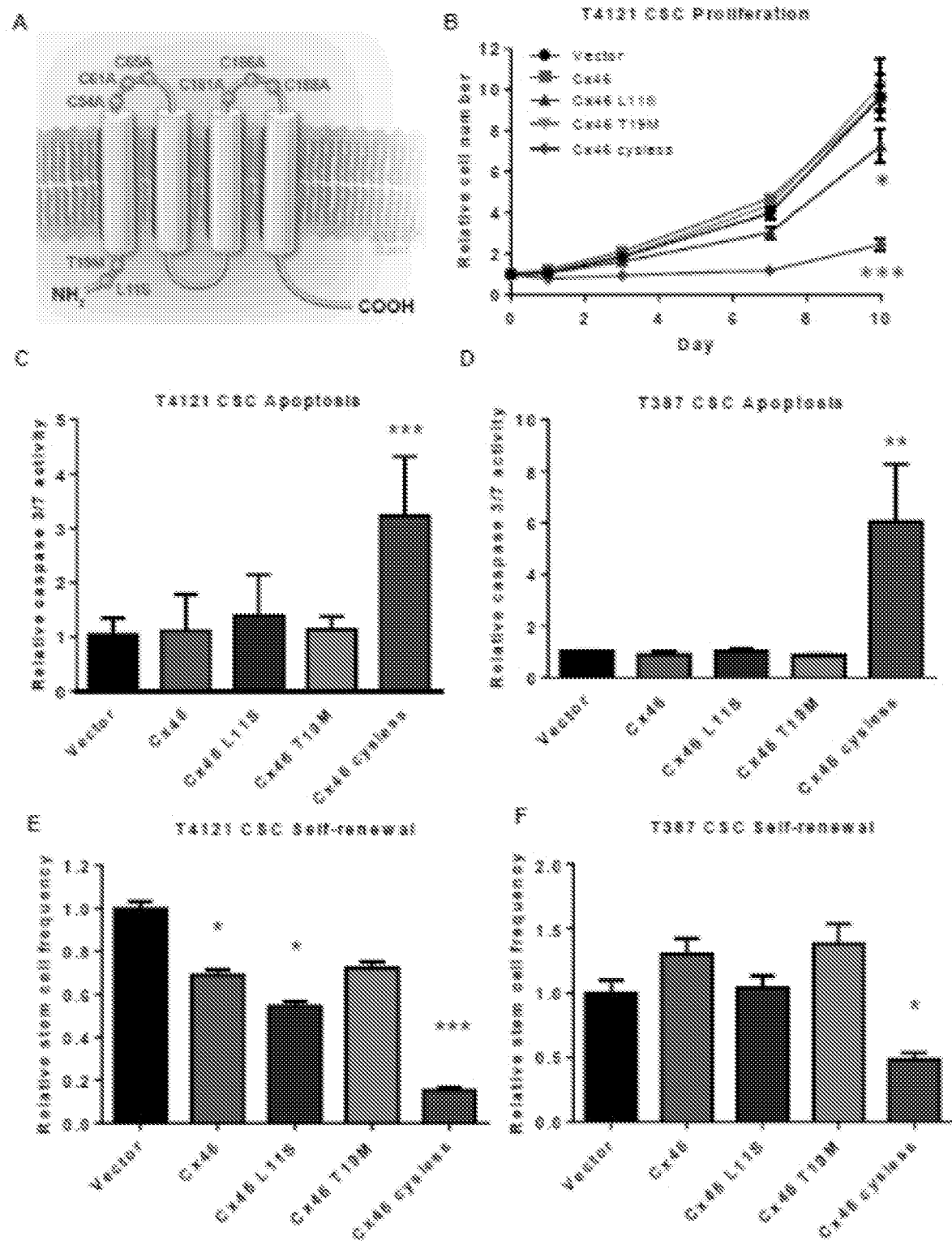


FIG. 2

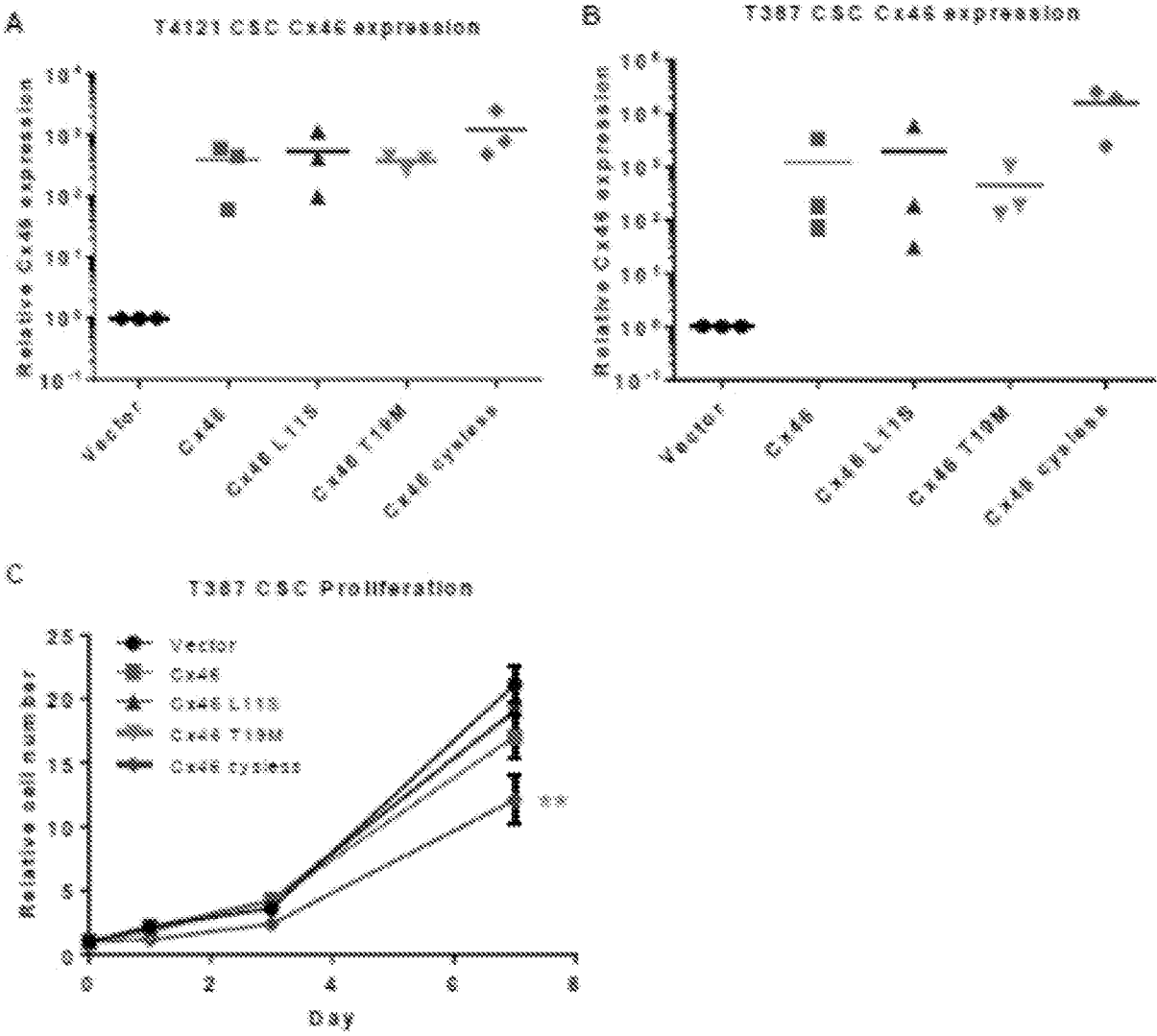


FIG. 3

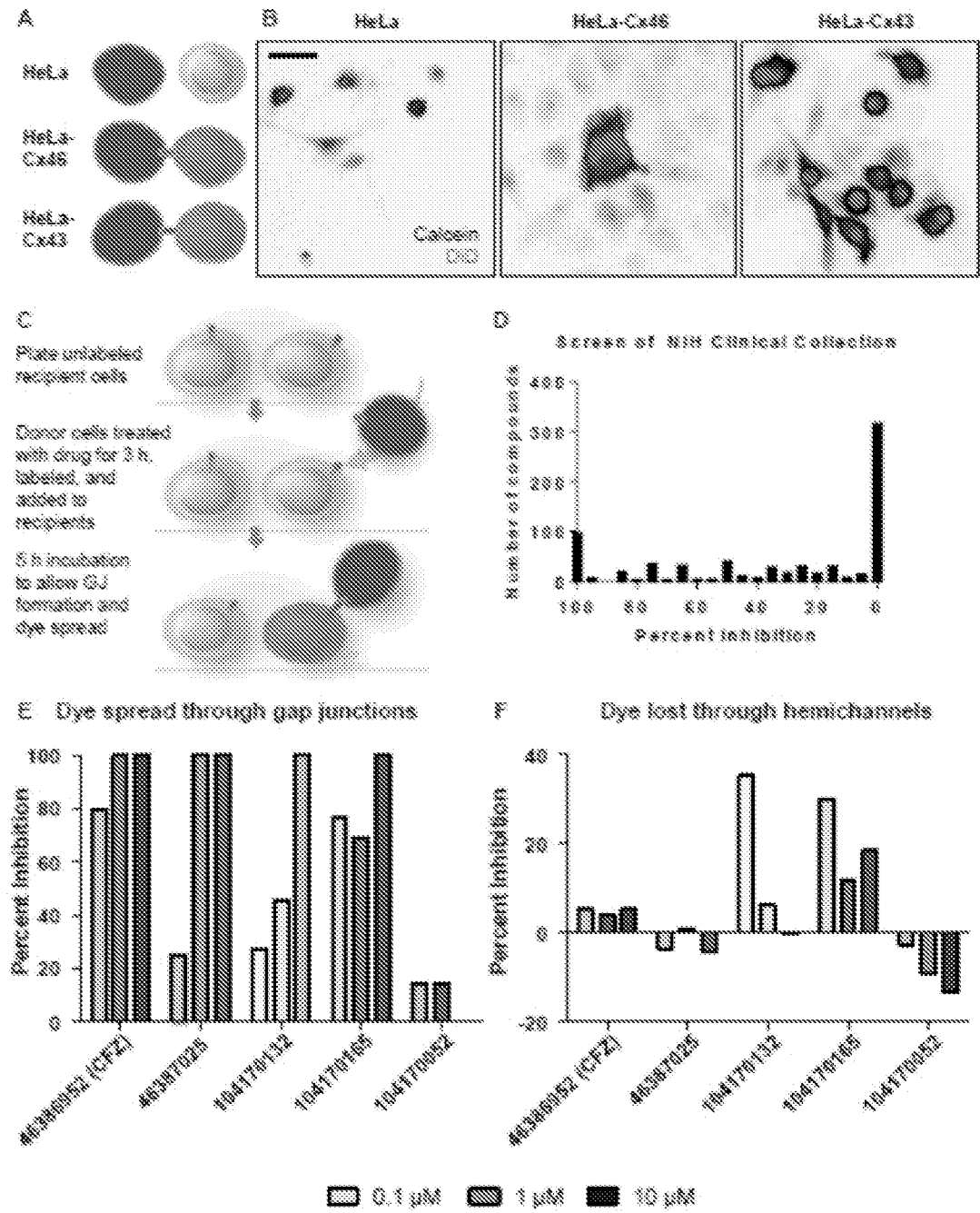


FIG. 4

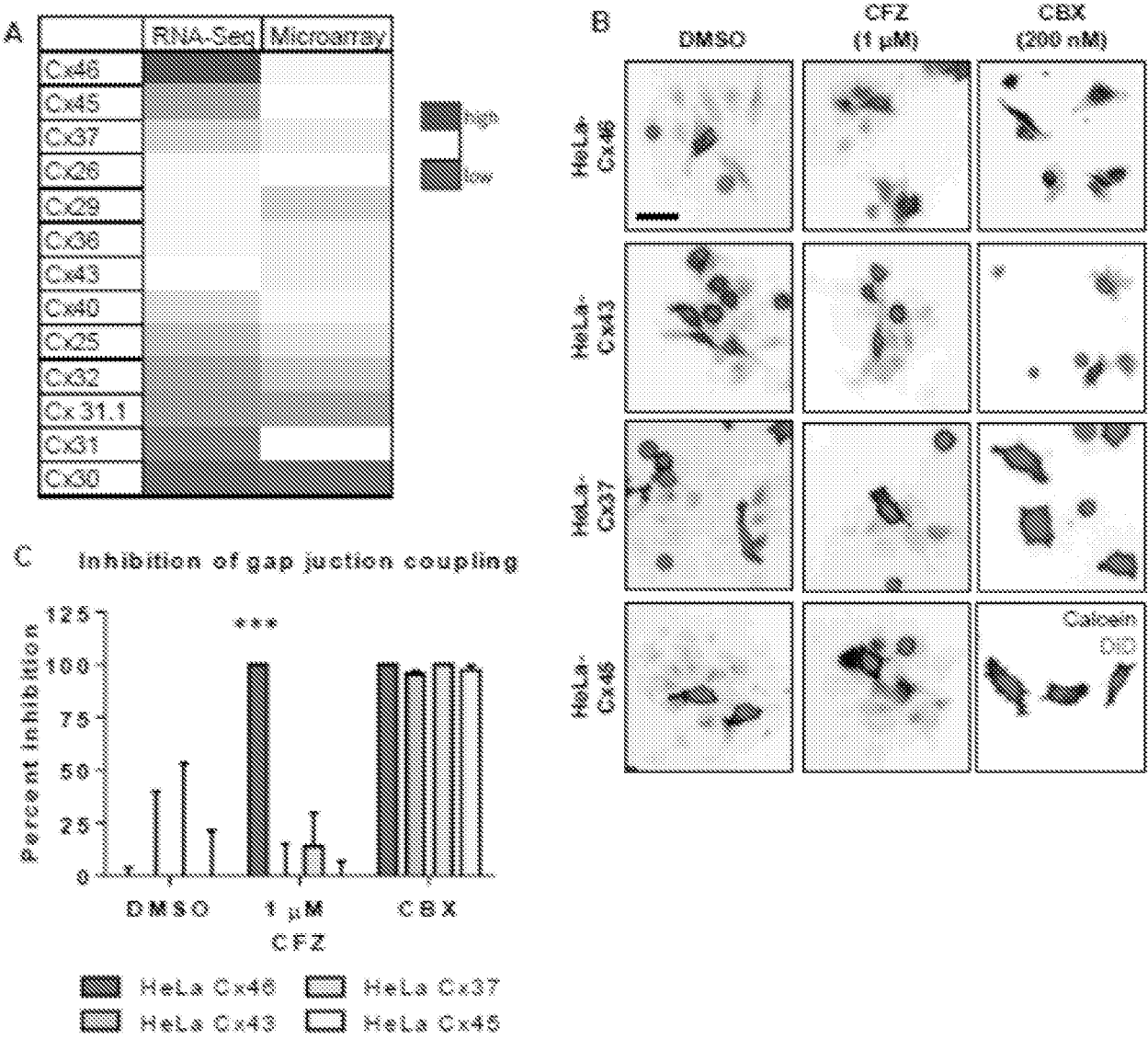


FIG. 5

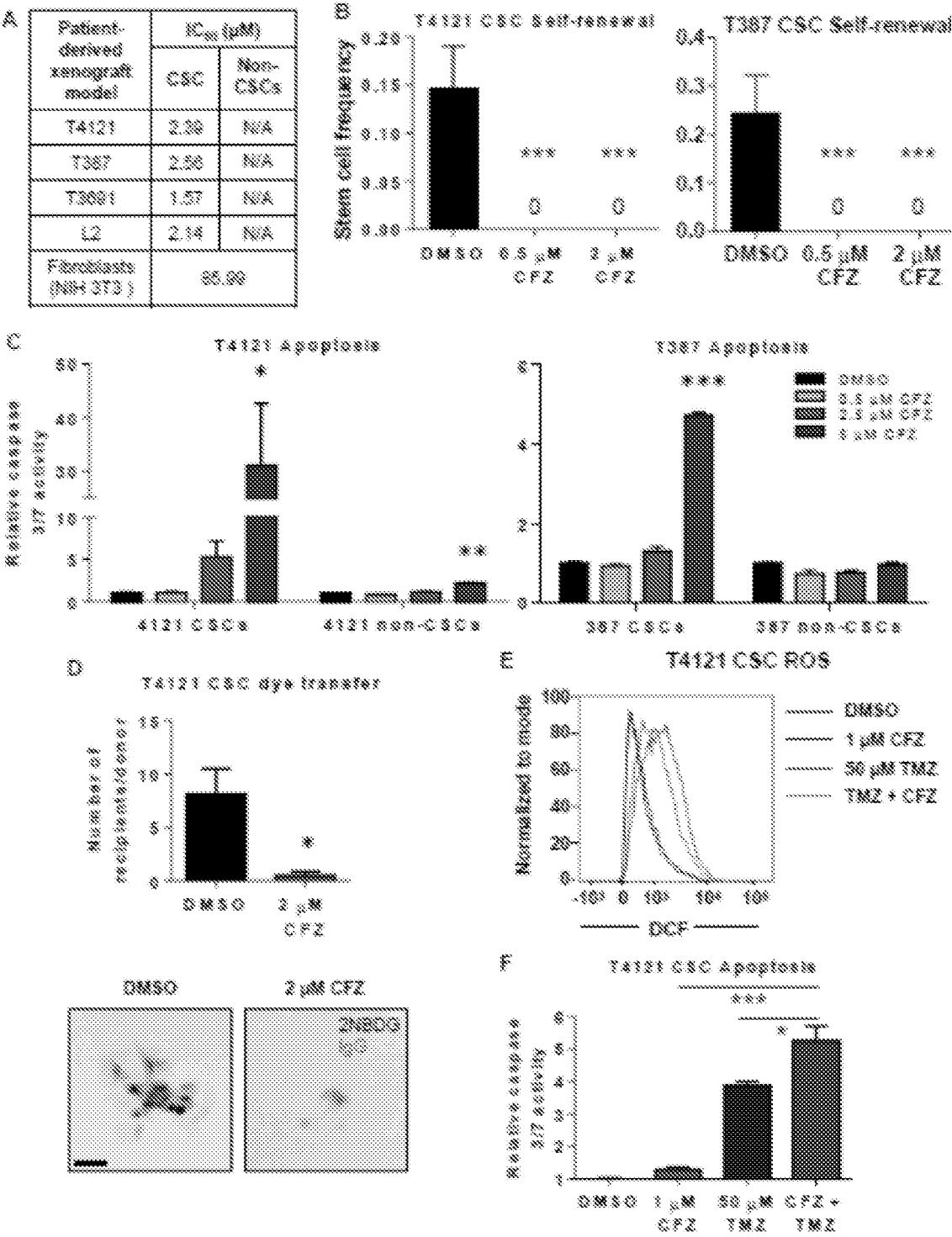


FIG. 6

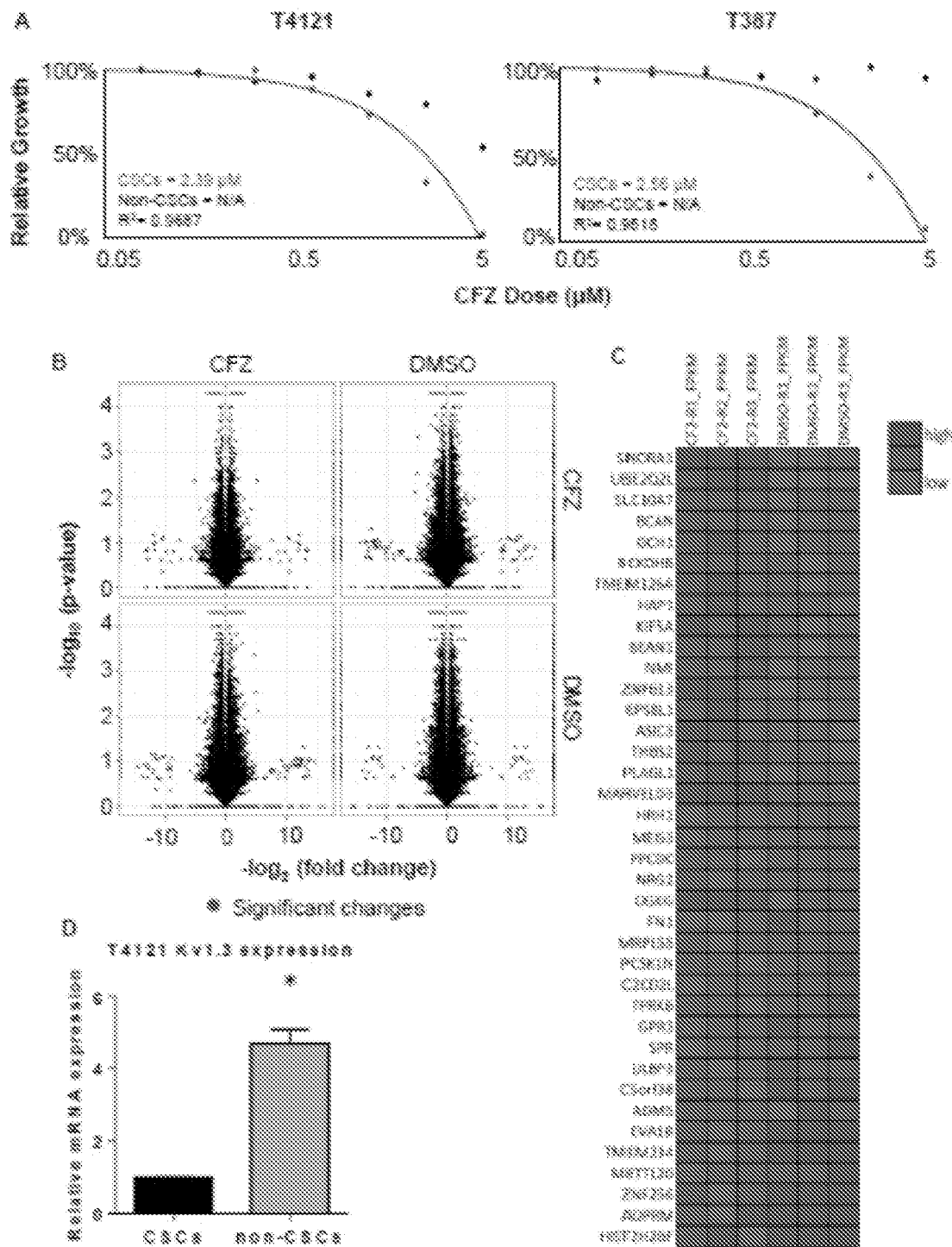


FIG. 7

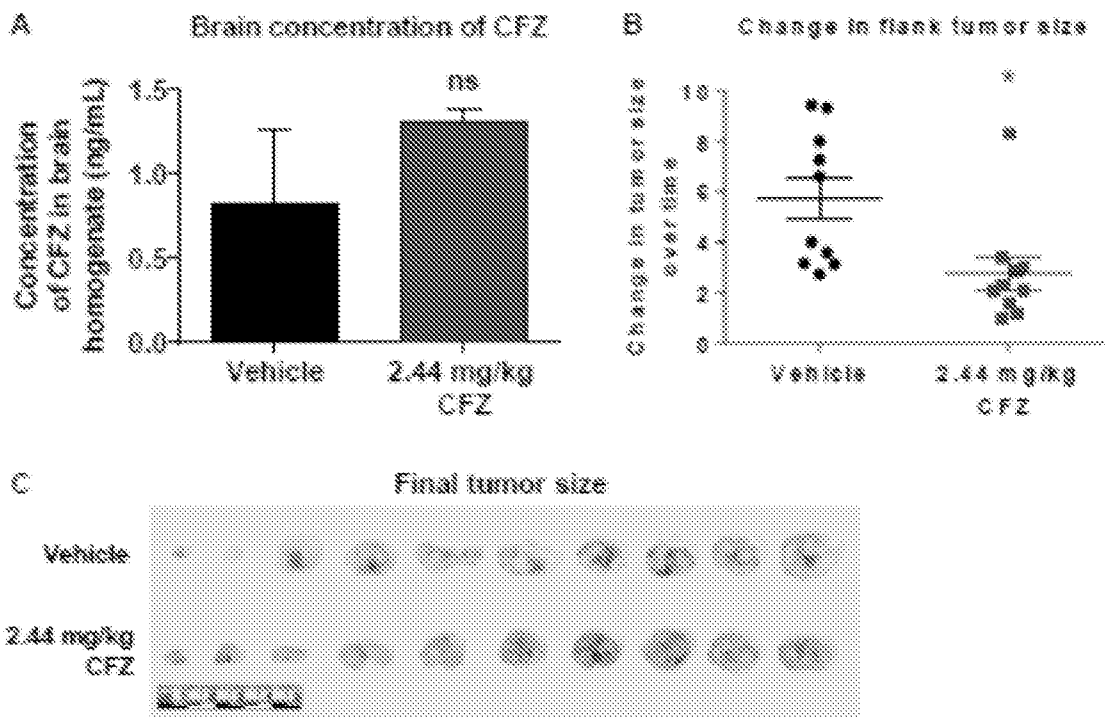
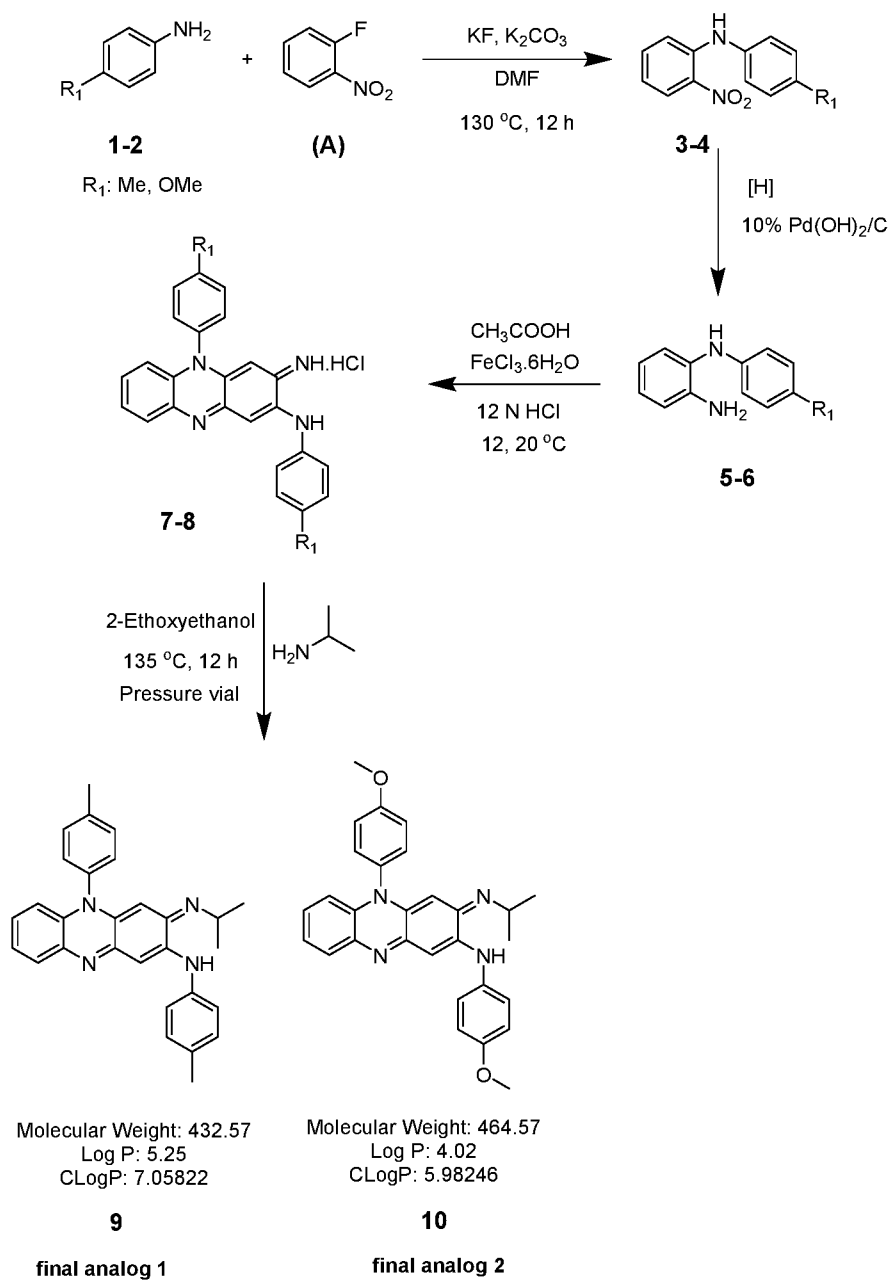


FIG. 8

Scheme 1



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/051742

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/282; A61K 31/337; A61K 31/4745 (2018.01)

CPC - A61K 9/0085; A61K 31/4745; A61K 31/555; A61K 33/24; A61M 25/0021 (2018.08)

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)

See Search History document

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

USPC - 424/649; 514/34; 514/49; 514/283; 514/449; 514/492 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 2016/0177298 A1 (AUCKLAND UNISERVICES LIMITED et al) 23 June 2016 (23.06.2016) entire document	1, 2, 7 ----- 8-18
X - Y	US 2004/0067952 A1 (MORRIS et al) 08 April 2004 (08.04.2004) entire document	19, 20, 23, 25-27 ----- 24
X - Y	US 2016/0143907 A1 (KAMTEK, INC.) 26 May 2016 (26.05.2016) entire document	28-30, 32-34, 36, 38
Y	US 2016/0166637 A1 (GOURDIE et al) 16 June 2016 (16.06.2016) entire document	8, 9, 15-18
Y	VENTURINI et al. "Targeting the Potassium Channel Kv1.3 Kills Glioblastoma Cells," Neurosignals, 01 September 2017 (01.09.2017), Vol. 25, No. 1, Pgs. 26-38. entire document	10, 11, 13, 14
Y	BRENNAN et al. "Clofazimine," Tuberculosis, 01 March 2008 (01.03.2008), Vol. 88, Iss. 2, Pgs. 96-99. entire document	12
Y	US 2015/0037437 A1 (RENISHAW PLC) 05 February 2015 (05.02.2015) entire document	18, 24
P, X	MULKEARNS-HUBERT et al. "Development of a Cx46 targeting strategy for cancer stem cells," bioRxiv, 26 March 2018 (26.03.2018). entire document. Article retrieved from the internet. URL: <https://www.biorxiv.org/content/early/2018/03/26/289397>	1-38

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

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

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

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

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Date of the actual completion of the international search

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/051742

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HITOMI et al. "Differential Connexin Function Enhances Self-Renewal in Glioblastoma," Cell Reports, 07 May 2015 (07.05.2015), Vol. 11, Iss. 7, Pgs. 1031-1042. entire document	1-38
A	BAIJNATH et al. "Evidence for the presence of clofazimine and its distribution in the healthy mouse brain," J Mol His, 25 July 2015 (25.07.2015), Vol. 46, Iss. 4-5, Pgs. 439-442 (only Pg. 439 provided). entire document	1-38